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Root Phosphomonoesterase as a Vital Component of Increasing Phosphorus Availability in Tropical Forests

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I am submitting herewith a dissertation written by Kristine Grace Manno Cabugao entitled "Root Phosphomonoesterase as a Vital Component of Increasing Phosphorus Availability in Tropical Forests." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Energy Science and Engineering.

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Root Phosphomonoesterase as a Vital Component of Increasing Phosphorus Availability in Tropical Forests

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Kristine Grace Manno Cabugao

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Dedication

For my family and friends who have never once faltered in their kindness and support.

For my fiancé who found ways to walk every step of this journey with me even from thousands of miles away.

For my co-advisors who constantly encouraged me to do my best and patiently edited every draft, presentation, and conference abstract sometimes despite their many deadlines.

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Abstract

Tropical forests, relative to other terrestrial ecosystems, exchange the largest amount of carbon with the atmosphere and also constitute a significant carbon sink. However, nutrient limitation, particularly of phosphorus (P), could limit growth of tropical forests and their function with the global carbon cycle. Thus, understanding root mechanisms to acquire P is necessary to representing the P cycle and corresponding interactions with plant growth. A large portion of total soil P in tropical forests occurs in organic forms, only accessible through root and microbial production of phosphatase enzymes. These phosphatase enzymes mineralize organic P into orthophosphate, the form of P most readily accessible to plants and microbes. My dissertation aims to understand the relationship between root and microbial phosphatase activity and available P in order to understand how phosphatase may contribute to soil P dynamics in tropical forests. In this work, I explore the variation of phosphatase with respect to tree species, soil depth, and elevated [CO_2].

I found that tree species and available P strongly influenced phosphatase produced by roots. Although bacterial community composition was most influenced by available P, phosphatase activity of bacteria was regulated instead by host tree species. Variation in phosphatase activity due to tree species could be due to differences in root or soil factors. I determined that the phosphatase activity released from roots (root phosphatase) is predicted by specific root length and available P while microbially-produced phosphatase enzymes in the soil (soil phosphatase) is more regulated by fine-root mass distribution and total soil P concentration. A major uncertainty is how phosphatase activity may respond in elevated [CO_2] conditions. In a greenhouse experiment, I found that root phosphatase increased in some tree seedlings exposed to elevated [CO_2], but not in others, perhaps due to P limitation. Ultimately, phosphatase activity represents a necessary function to create a source of available P from soil organic P compounds. Phosphatase activity contributes to the capacity to intensely use sources of P from the rhizosphere representing an important facet of P acquisition, different, but complementary to root traits that increase root exploration of the soil volume.

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Chapter 1

Introduction

Tropical forests constitute a sizeable carbon sink and are unparalleled in the magnitude of carbon, energy, and water they exchange with the atmosphere (Santiago, 2015). However, soils beneath tropical forests are often highly weathered and devoid of phosphorus (P) (Walker & Syers, 1976) – a crucial nutrient for nucleic acid synthesis, plant metabolism, and phospholipid membranes (Raven, 2015). An increasingly recognized constraint in understanding tropical forest structure, composition, and response to atmospheric and climatic change is the capacity of the root system to alter form and function to increase P acquisition.

The overarching goal of the Next Generation Ecosystem Experiments - Tropics (NGEE-Tropics) project is to improve how tropical forests are represented in global ecosystem models. One focus to achieve this goal is improving our understanding and model representation of the phosphorus cycle and its feedbacks on forest productivity (Chambers et al., 2014). While the availability of P within soils is governed by many factors, among the most significant to plant uptake is the extent to which roots can access available P via adaptation or changes in root distribution, symbiosis with mycorrhizae, and the capacity to increase production of root phosphomonoesterase. Meeting plant demand in part relies on root- and microbially- produced phosphomonoesterase enzymes (in models, biochemical mineralization) that break down organic P compounds to produce orthophosphate (Yang, Thornton, Ricciuto, & Post, 2014), given the low availability of the form of inorganic P (orthophosphate) necessary for plant growth (Reed et al., 2011). The important role of P in constraining growth of tropical trees highlights the importance of the capacity of the root system to acquire P to understanding tropical forest productivity and subsequent carbon sequestration (Fernández-Martínez et al., 2014).

My dissertation addresses NGEE-Tropics goals by exploring how root and microbial phosphomonoesterase varies between tree species and sites with differing phosphorus availabilities (Chapter 2), connecting root and soil phosphomonoesterase to root distribution and soil characteristics – parameters already present in ecosystem models (Chapter 3), and examining the potential of root phosphomonoesterase and microbial communities to change in response to elevated CO_2 (Chapter 4).

Tropical forests and the global carbon cycle

Across all terrestrial ecosystems, no other exchanges as much carbon, energy, and water as tropical forests (Santiago, 2015). Tropical forests span major regions in tropical America, Africa, Southeast Asia, Madagascar, and New Guinea, with significant portions in Australia and numerous tropical islands between the Tropics of Cancer and Capricorn (Malhi et al., 2014; Sheldon, 2019). Each year tropical forests account for approximately 35% of global net primary productivity (Cernusak et al., 2013; Reed et al., 2011), storing about 25% of all terrestrial carbon (C) in biomass and soil (Zuidema et al., 2013), and half of all plant biomass in the terrestrial biosphere (Cernusak 2013). Historically, the land surface has been considered a strong global carbon sink, with a huge portion of that sink residing in soils and the living biomass of tropical forests (Brienen et al., 2015). Within the Amazon alone is an estimated 150-200 Pg C (Brienen 2015). Because tropical forests capture a significant proportion of the carbon released from anthropogenic activities, thereby influencing the pace of CO_2 accumulation in the atmosphere (Yang et al., 2014; Sheldon 2019), how tropical forests respond to future climatic conditions and the factors that mitigate those responses carry significant consequences for the global carbon cycle.

Generally speaking, models predict that elevated [CO_2] will increase growth of tropical forests - CO_2 fertilization (Cernusak 2013). However, while some studies have indicated an enhanced tropical carbon sink due to increased photosynthesis (Keenan et al., 2016), others have suggested a declining tropical sink (Brienen et al., 2015) due to increasing tree mortality. Keenan et al. (2016) found an increase in carbon uptake using satellite observations of aboveground growth and a set of 10 dynamic global vegetation models. However, a 30-year study of biomass dynamics in the Amazon using 321 plots demonstrated a long-term decline in carbon accumulation by 1/3 due to decreasing growth rates and increasing biomass mortality (Brienen et al., 2015). Similarly, a 12-year study using NASA Moderate Resolution Imaging Spectroradiometer (MODIS) data of pantropical satellite observations of net annual aboveground carbon density found that tropical forests acted as a source of carbon, emitting 425.2 ± 92.0 Teragrams of C per year (Baccini et al., 2017). Furthermore, a study of how different ecosystem models simulate tropical forest productivity indicated wide variability in predictions, suggesting an incomplete understanding of the factors that influence tropical forest response to atmospheric and climatic change (Cavaleri, Reed, Smith, & Wood, 2015). The introduction nitrogen (N) and

more recently, P, in ecosystem models have only served to reduce simulated CO_2 uptake of forests (Wang, Law, & Pak, 2010; Goll et al., 2012; Yang et al., 2014). Thus, capturing tropical forest response will require improving how the phosphorus cycle in tropical forests is represented in terrestrial biosphere models (Reed, Yang, & Thornton, 2015).

Phosphorus limitation in tropical forests

Nitrogen and phosphorus generally limit plant productivity across the globe (Aerts & Chapin, 1999), though phosphorus is largely considered to limit processes in vast areas of tropical forests. Unlike nitrogen, which can accumulate due to the activity of N2-fixing bacteria; phosphorus is finite, derived primarily from the weathering of underlying rock (parent material) (Reed 2011). Walker and Syers (1976) proposed that as ecosystems age, there would be increasingly smaller amounts of inorganic P available for plant uptake, with the majority of phosphorus existing either as organic P compounds or in forms that were biologically unavailable - occluded P. Tropical forests occur on some soils that have experienced millions of years of weathering (Sayer & Banin, 2016). Furthermore, high temperatures and rainfall encourage within tropical forests promote rapid weathering (Reed 2011). Thus, in some tropical forests, high rates of weathering over millions of years has resulted in large areas of tropical forests occurring on Oxisol and Ultisol soils characterized by low P availability (Reed 2011). Indeed, recent global phosphorus surveys indicate that much of the original primary mineral P in Oxisol and Ultisol soils were in the form of organic P or occluded P, rather than the form of phosphorus that can be readily taken in by plants - orthophosphate (hereafter, available P; Yang 2011). What little proportion of available P for plant uptake remains is subject to sorption process due to the high the high presence of 1:1 clays and aluminum and iron oxides that strongly bind available P reducing its availability for plant uptake (Reed 2011).

Evidence that P limits plant growth stems primarily from natural gradient, leaf and litter, and fertilization studies, though the evidence is mixed with some studies concluding P limitation and not in others. The Hawai'ian Long Substrate Age Gradient (LSAG) is made up of six sites that collectively form a spectrum of forest age from 300 years to 4.1 million years (Sayer and Banin 2016). Consistent with the theory posed by Walker and Syers (1976), the oldest sites have the lowest concentrations of available P, limiting productivity (Sayer and Banin 2016). Furthermore, increasing P availability across this gradient corresponded with increased tree diameter growth (Santiago 2015). Leaf and litter studies comparing temperate and tropical leaves

indicate more efficient use of P per unit biomass due to higher C:P ratios in tropical leaves, as well as higher P resorption evident in higher N:P ratios in litter (Reed 2011). Nutrient fertilization studies are mixed in their conclusions, though long-term studies do suggest P limitation of primary production (Reed 2011). For example, P fertilization did increase growth, but only in small trees (Alvarez-Clare & Mack, 2015), and growth responses were only detected when both N and P were added (Santiago 2015). Understanding root responses to P availability will be a crucial facet of determining plant responses to P limitation given that roots are the primary organs for water and nutrient uptake.

Root traits and phosphorus acquisition

Roots mediate critical exchanges between plants and soil processes, regulating feedbacks that influence decomposition, soil organic matter formation, and nutrient cycling (Weemstra et al., 2016). Roots are often excluded from ecosystem model (Warren et al., 2015), despite their importance to understanding ecosystem function. However, phosphorus uptake, as with other nutrients, occurs at the root surface. Thus, characterizing fine roots and understanding their function is critical to describing and modeling how roots influence phosphorus uptake and cycling in tropical forests. Ultimately, understanding the potential of phosphorus to limit tropical forest growth in elevated CO_2 conditions requires determining the degree to which roots can increase P acquisition.

Root traits are generally defined as root characteristics that inform our understanding of plant growth, reproduction, or survival (Violle et al., 2007; McCormack et al., 2017). Traits critical to increasing P uptake can be categorized into traits that enhance acquisition by increasing the soil volume explored for patches of available P and those traits that increase P availability within the rhizosphere. For example, changes to root morphological and architectural traits are among the first responses to P limitation. Increased specific root length and root allocation at shallower depths where available P concentration both serve to enhance, he exploration of soil volume (Drewniak, 2019; Hodge, 2004; Lambers, 2006; Lambers, Raven, Shaver, & Smith, 2008). Furthermore, formation of mycorrhizal symbiosis extends root exploration because the fungi hyphal network extends across great distances, transporting phosphate ions to the root much faster than the diffusion of phosphate to the root surface (Clarkson 1985). Root traits that increase P availability include root exudation of carboxylates like citrate and malate, which increase P availability by binding to metal cations that remove

orthophosphate out of solution and displacing phosphate from soil surfaces through ligand exchange (Lambers 2006). The release of carboxylates also mobilizes organic P, the substrate for phosphatase enzymes that are capable of increasing P availability by breaking them down to release inorganic P.

Phosphatase and P availability in the rhizosphere

While the release of carboxylates mobilizes available P and organic P, it is the release of phosphatase enzymes that catalyzes the mineralization of organic P into available P within the rhizosphere. Phosphatase enzymes exuded by roots (root phosphatase) and phosphatase from microbes released into the soil (soil phosphatase) are functionally the same, though difficult to trace to the source once released into the soil environment. However, it is generally accepted that phosphatase measured at the root surface is largely of root-origin, though it is likely to encompass some phosphatase enzymes of microbial-origin too unless the plant is grown aseptically (Skujins, 1978). Similarly, phosphatase measured in soils likely contains some enzymes from the root as well (Skujins, 1978). Nonetheless, the observation that plants could only acquire inorganic nutrients from soil led to the theory that mineralization of organic P by phosphatase enzymes to release orthophosphate was a critical mechanism of P nutrition (Tarafdar & Jungk, 1987). Since then, the recognition of phosphatase as a vital component of accessing the soil organic P pool has only increased, spurred by the significant correlation between the depletion of organic P at the root surface and phosphatase activity in wheat and clover (Tarafdar & Jungk, 1987), and the strong localization of phosphatase within 1.2 - 1.6 mm of the root surface (Tarafdar & Claassen, 1988). Furthermore, concurrent ^{32}P-labeled uptake and phosphatase studies confirm a strong positive correlation between phosphatase and P uptake (Lee 1982), and P depletion studies in tomato indicated that the release of phosphatase was proportional to the decline of shoot P concentrations below 0.25% of dry weight (Speir & Cowling, 1991). Altogether, these early greenhouse and crop plant studies have assembled a portrait of phosphatase as an important response to declining P content within the shoot by enabling P uptake through the depletion of organic P within the vicinity of the root surface where P uptake occurs.

Within tropical forest soils, organic P can comprise a significantly larger proportion of total soil P than available P (George et al., 2011; Turner & Engelbrecht, 2011). Thus, the recycling of organic P as mediated by phosphatase enzymes produced by plants and microbes is

critical to supplying orthophosphate for plant uptake (Kitayama, 2013; Malcolm, 1983; Rao et al., 2000). Phosphatase is a broad term encompassing a group of enzymes capable of hydrolyzing various organic P compounds, which in tropical soils, occur as a heterogenous mix of phosphodiesters (nucleic acids, phospholipids), anhydrides (ADP), and phosphomonoesters (sugar phosphates, mononucleotides, and inositol phosphates) (Eivazi & Tabatabai, 1977; Condron, 2005). Phosphodiesterase (EC 3.1.4) breaks down phosphodiesters into phosphomonoesters, the largest fraction of soil organic P. Next phosphomonoesterase (EC 3.1.3; PME) mineralizes phosphomonoesters, resulting in available P (Turner & Haygarth, 2005). Although the association of phosphomonoesterase with soil organic P cycling is well established, the majority of these studies occur in temperate ecosystems in bulk soil (i.e., soil collected without considering proximity to roots) or, when the rhizosphere is considered, in controlled greenhouse studies on crop plants. However, plant P uptake relies heavily on processes occurring within the rhizosphere, specifically root traits and the production of phosphomonoesterase. Therefore, placing phosphomonoesterase in the context of roots and the rhizosphere is crucial to understanding and modeling the role of root-mediated P acquisition in regulating tropical forest growth.

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Chapter 2

Root and Rhizosphere Bacterial Phosphatase Activity Varies with Tree Species and Soil Phosphorus Availability in Puerto Rico Tropical Forest

My use of "we" in this chapter refers to my co-authors and myself. This chapter is a lightly revised version of a paper of the same title, authored by Kristine Grace M. Cabugao, Collin M. Timm, Alyssa A. Carrell, Joanne Childs, Tse-Yuan S. Lu, Dale A. Pelletier, David J. Weston, and Richard J. Norby *published* in *Frontiers in Plant Science* on October 30, 2017.

Abstract

Tropical forests generally occur on highly weathered soils that, in combination with the immobility of phosphorus (P), often result in soils lacking orthophosphate, the form of P most easily metabolized by plants and microbes. In these soils, mineralization of organic P can be the major source for orthophosphate. Both plants and microbes produce phosphatases capable of mineralizing a range of organic P compounds. However, the activity of these enzymes depends on several edaphic factors including P availability, tree species, and microbial communities. Thus, phosphatase activity in both roots and the root microbial community constitute an important role in P mineralization and P nutrient dynamics that are not well studied in tropical forests. To relate phosphatase activity of roots and bacteria in tropical forests, we measured phosphatase activity in roots and bacterial isolates as well as bacterial community composition from the rhizosphere. Three forests in the Luquillo Mountains of Puerto Rico were selected to represent a range of soil P availability as measured using the resin P method. Within each site, a minimum of three tree species were chosen to sample. Root and bacterial phosphatase activity were both measured using a colorimetric assay with para-nitrophenyl phosphate as a substrate for the phosphomonoesterase enzyme. Both root and bacterial phosphatase were chiefly influenced by tree species identity. Though tree species was the only significant factor in root phosphatase activity, there was a negative trend between soil P availability and phosphatase activity in linear regressions of average root phosphatase and resin P. Permutational multivariate analysis of variance of bacterial community composition based on 16S amplicon sequencing indicated that bacterial composition was strongly controlled by soil P availability (p-value < 0.05). These results indicate that although root and bacterial phosphatase activity were influenced by tree species; bacterial community composition was chiefly influenced by P availability. Although the sample size is limited given the tremendous diversity of tropical forests, our study indicates the importance of roots and bacterial function to understanding phosphatase activity. Future work will broaden the diversity of tree species and microbial members sampled to provide insight into P mineralization and model representation of tropical forests.

Introduction

Tropical forests annually accumulate the highest amount of biomass among terrestrial ecosystems, constituting a significant global carbon sink. In 2011, tropical forests were estimated to have stored 262.1 Pg C in living above- and belowground biomass. In contrast, boreal forests contained 53.9 Pg C and temperate forests, 46.6 Pg C (Pan et al., 2013). Paradoxically, many tropical forests occur on highly weathered soils with lower amounts of total P and a higher fraction of occluded (and unavailable) P (Yang et al., 2013). Although both nitrogen (N) and P are likely to co-limit natural ecosystems, P is of particular interest because soil P cannot be replenished biologically in the way that N fixation, prevalent in the tropics, can enhance available N (Cleveland et al., 2011; Townsend et al., 2011). Orthophosphate (H2PO4- and HPO42-) is the form of P most readily available for plant and microbial uptake, vital for many compounds involved in energy transfer, metabolism, membrane transport, signaling, and in the formation of nucleic acids (Raven, 2015). However, prolonged warm and moist climate of the tropics encourage high weathering rates, which deplete sources of P. In addition, P forms insoluble complexes with aluminum and iron ions, both of which are considered geochemical sinks for phosphate due to their specific surface area (Chacon et al., 2006). High weathering rates and the strong reaction between soil iron and aluminum to phosphate result in tropical soils typically devoid of orthophosphate (Vitousek and Sanford, 1984; Chacon et al., 2006; Dalling et al., 2016). Therefore, it is critical to understand factors that regulate P availability given that P limitation could modulate important tropical ecosystem properties such as photosynthetic activity (Bloomfield et al., 2008), interactions with the N cycle, belowground C cycling, litter decomposition, soil organic matter turnover (Cleveland et al., 2011), growth rate of young trees and seedling survival (Alvarez-Clare et al., 2013), respiration (White and Hammond, 2008), tree distribution (Condit et al., 2013), and microbial biomass and composition (Liu et al., 2012). The immense diversity and vitality of tree species in tropical forests suggests the existence of multiple mechanisms that can alleviate limitations in soil P supply (Lynch and Brown, 2001; Lambers et al., 2006). Although interactions between roots and microbes are important to regulating P acquisition, the correlation between root and microbial traits and their influence on soil P are not well understood (Marschner et al., 2011; Mommer and Weemstra, 2012).

The plant microbiome plays an active role in shaping root and soil characteristics that modulate plant P acquisition by altering existing pathways and providing new biochemical

capabilities (Bulgarelli et al., 2013). P uptake occurs through root hairs, the root epidermis, and mycorrhizal hyphae, effectively restricting P uptake to the soil volume closest to the root surface and mycorrhizal hyphae (Schachtman et al., 1998). However, both arbuscular mycorrhizal fungi (AMF) and plant growth-promoting bacteria influence this process in a variety of ways. While there has been much attention in relating mycorrhizae to root traits with regards to P acquisition (Valverde-Barrantes et al., 2013; Comas et al., 2014; Kong et al., 2014), less attention has focused on bacterial members of the microbial community and the ways in which they also shape the root system. Many members in the plant growth promoting bacteria group can alter root architecture and morphology by synthesizing major plant hormones such as auxin and ethylene, stimulating the formation of lateral roots, decreasing primary root length, and increasing root hairs – indirectly influencing the effectiveness of soil foraging (Bulgarelli et al., 2013; Niu et al., 2013; Vacheron et al., 2013). In addition, bacteria also directly enhance P availability in the immediate vicinity of the root. P occurs in both inorganic and organic forms. Orthophosphate availability from minerals (inorganic) is highly dependent on soil pH and by soil processes such as sorption–desorption and dissolution– precipitation. Bacteria are capable of solubilizing P from these minerals by the release of protons and secondary metabolites, enhancing P availability in the rhizosphere directly (Bunemann et al., 2004; Vacheron et al., 2013). However, in tropical regions, mineralization of organic P is the major source for orthophosphate (Vitousek and Sanford, 1984).

Plants and microbes release phosphatase enzymes to mineralize organic P compounds. Phosphatase activity refers to the actions of two complementary, but distinct enzymes: phosphodiesterase (PDE) and phosphomonoesterase (PME). PDE hydrolyses complex organic P compounds such as nucleic acids and phospholipids into phosphomonoesters (mononucleotides and inositol phosphates). PME further mineralizes these compounds into orthophosphate which can be directly absorbed by plants and microbes (Rejmnkov et al., 2011; Stone and Plante, 2014). These extracellular enzymes are key agents in organic P mineralization and play important roles in plant response to limited P availability or increasing P demand (Dakora and Phillips, 2002; Burns et al., 2013; Dalling et al., 2016). More recently, phosphatase activity has been determined to be a crucial uncertainty in modeling P cycling in ecosystem models because P mineralization is among the least understood aspects of P dynamics (Reed et al., 2015). Several modeling studies suggest that the magnitude of phosphatase activity could significantly influence

CO_2 uptake in tropical forests (Goll et al., 2012; Yang et al., 2016). Determining the interaction between roots and the bacterial community in regulating phosphatase activity and the factors that influence those interactions is central to understanding P mineralization. However, much of what we currently understand about the role of bacteria in P acquisition is derived from agricultural studies. Although these studies have provided valuable insight, there is a need to extend understanding of those systems to tropical forests, especially with respect to root traits and function (Richardson and Simpson, 2011; Pii et al., 2015). The improved understanding of variations among different tree species of root and bacterial function may provide a means to improve representation of tropical forests in ecosystem models and help us gain a more thorough understanding of how roots and their bacterial community enable tropical forests to thrive in severely limited P soils.

To understand how root and bacterial function differed by tree species and soil P availability, we assessed phosphatase activity in roots and bacterial isolates. Because bacterial community composition may influence this function, we also characterized the bacterial component of the microbial community by sequencing the 16S amplicon. The overarching question was how soil P availability and tree species influenced root and associated bacterial phosphatase activity, as well as root associated bacterial community composition. We hypothesized that (1) phosphatase activity would be higher in areas with low P availability, (2) within a site, tree species would differ in phosphatase activity, and (3) bacterial community composition would primarily depend on tree species. The numerous interactions between roots and the bacterial community determine, in part, P mineralization of tropical ecosystems. Therefore, understanding factors which influence those interactions provides a means to unravel how rhizosphere processes belowground relate to the forest above.

Materials and Methods

Study sites and tree species

To examine the influence of P availability on root and bacterial traits, three study sites in the Luquillo mountains of northeastern Puerto Rico (18º30'N, 65º80'W) were chosen to represent a spectrum of P availability. Previous measurements of Rio Icacos(Icacos), El Verde Ridge (Ridge), and El Verde Valley (Valley) indicated lower total P in Icacos and higher total P in Ridge and Valley reflecting differences in parent material. Icacos, formed on quartz-diorite parent material had the lowest total P content (170 ± 25 ppm P) followed by Ridge (290 ± 15

ppm P) and finally, Valley (410 ± 43 ppm P), both of which were on volcaniclastic parent material known to contain higher amounts of soil P (Mage and Porder, 2013). In Icacos, Ridge, and Valley, the most common tree species were sampled for root phosphatase. In Icacos, the tree species sampled were *Cecropia schreberiana* Loefl., *Micropholis garcinifolia* Grisseb, *Cyrilla racemiflora* L., and *Prestoea montana* Hook. In Ridge and Valley, the trees sampled were as follows: *Prestoea montana* Hook, *Dacryodes excelsa* Vahl, and *Manilkara bidentata* Chevalier.

Soil collection and resin P assay

At each site, nine cores (2.5 cm diameter) were collected from 0 to 10 cm soil depths to measure site level P availability using the resin P method (Calvert et al., 1993). The resin P method estimates the amount of labile P available for plant and microbial uptake by imitating root removal of phosphate from the soil solution. Charged resin membrane strips in sample soil suspensions attract phosphate ions. Then, the resin strips are washed to remove adhering phosphate ions, resulting in an extract that serves as a proxy for orthophosphate availability in the soil (Van Raij et al., 2009). From each core, 8 g of soil fresh weight were mixed with 80 mL of distilled water and five resin strips charged with sodium bicarbonate. Samples were shaken for 24 hr after which the resin strips were washed of the adsorbed phosphate ions in 50 mL of sulfuric acid. Orthophosphate in the resulting extract was quantified using the Lachat QuikChem 8500. Site level P availability is presented as an average of the nine cores taken at each site.

Root clusters

The first three most distal ends of the root were collected for the root phosphatase assay according to recent studies that indicate functional differences in the root system. The first three distal ends (orders) are associated with resource acquisition in contrast with higher orders which are involved in resource transport (McCormack et al., 2015). Three root samples consistent of the first three orders from the most common tree species in each site were sampled during November 2015. Root samples were collected by tracing roots from the base of each individual tree and gently excavating to the terminal three root orders. In total, 12 root samples were collected from Icacos, 9 root samples from Ridge, and 9 root clusters from Valley (n = 3 per tree species). Collected roots were kept cold during transport using cold packs and shipped overnight to Oak Ridge National Laboratory for further analyses.

Bacterial strain isolation

To characterize Ridge and Valley bacterial diversity, bacterial isolates and community

composition were collected during August 2015. Adhering soil from roots of *D. excelsa* and *P. montana* collected at Ridge and Valley were washed off using 10 mM magnesium sulfate. The resulting slurry was pooled for each tree species at each site before taking three serial dilutions. Each dilution was plated onto solid R2A media (18.2 g Difco^{TM} R2A L^{-1}) (Reasoner and Geldreich, 1985). Throughout 1 week, colonies were selected and re-streaked for isolation on fresh R2A solid agar plates. Single colonies of the resulting plates were re-streaked on fresh R2A solid agar plates three times to ensure pure colonies of each isolate. Bacterial stocks of each isolate were made by growing individual colonies overnight in R2A liquid medium at 25 ºC and mixing the ensuing bacterial culture with an equal volume of 50% glycerol. These solutions were frozen in -80 ºC and re-streaked onto fresh plates for downstream functional assays.

Microbial community

Culturing bacteria enables insight into bacterial function of members of the bacterial community. In addition, a broader survey of the bacterial community via 16S amplicon sequencing provides the opportunity to understand which phyla are present in the bacterial community, placing into context observed bacterial function. Therefore, rhizosphere soil from Ridge and Valley was collected from *D. excelsa* and *P. montana* roots for bacterial 16S rRNA gene sequencing. Tweezers were used to separate adhering soil from terminal roots. Approximately 50 mg of those roots were washed in 50 ml falcon tubes with milliQ H2O before adding rhizosphere soil and pelleting the samples from each tree in Ridge and Valley. A total of 12 samples for DNA extraction from D. excelsa and P. montana roots were collected (three individuals surveyed per species per site). DNA was extracted from the soil pellet using MoBio PowerSoil kit as per manufacturer's instructions (Mo Bio Laboratories) and purified using the Zymo clean-up kit (Zymo Research). Samples were then stored in -20 ºC. Prior to amplification, DNA concentration was normalized to 10 ng ml^{-1}.

Root phosphatase

To estimate quantitative phosphorous transformation, phosphomonoesterase (PME) and phosphodiesterase (PDE) activities were measured for the most common tree species at Icacos, Ridge, and Valley. A modified version of the colorimetric soil phosphatase test by Tabatabai and Bremner (1969) was used for root samples with para-nitrophenyl phosphate (pNPP) and bis-para-nitrophenyl phosphate (bis-pNPP) as a substrate for PME and PDE activities, respectively (Dr. Benjamin L. Turner, personal communication). Enzyme activity for tree species was

calculated as the average of all technical replicates for each biological replicate of each species (n = 9). Approximately 0.3 - 0.1 g of roots were weighed with minimal cutting to maintain natural root surface area. Weighed roots were placed between moistened paper towels to prevent drying during the weighing process for other samples. Each weighed root was then placed into a glass vial with 9 mL of 50 mM sodium acetate (pH D 5.0). Following a 5-min equilibration time in a 27 ºC shaker, 1 mL of 50 mM pNPP was added for the PME assay, or 1 mL of 50 mM bis-pNPP for the PDE assay. Samples were incubated in the shaker for 1 hr, and then 0.5 mL of the sample solution was added to 4.5 mL of 0.11 M NaOH to terminate the reaction. Blank solutions were pure 10 mL of 50 mM sodium acetate incubated along with the samples, 0.5 mL of which was added to 4.5 mL of 0.11 M NaOH. Absorbance at 410 nm was read on a Thermo Spectronic 20D and compared to a standard curve generated from para-nitrophenol (pNP), the yellow end-product released from PME or PDE hydrolysis of pNPP.

Culture of bacterial isolates and bacterial phosphomonoesterase activity

Bacterial PME activity was assessed in isolated strains to estimate potential contribution of bacteria to P mineralization. Each bacterial strain was grown in 1.5 mL R2A overnight before centrifugation at 10,000 rpm for 20 min. A total of 1 mL of the resulting supernatant was added to 1 mL of 50 mM pNPP and 4 mL of modified universal buffer (MUB). MUB consisted of (for 1 L): 12.1 g Tris(hydroxymethyl)aminomethane (THAM or Tris base); 11.6 g maleic acid; 14.0 g citric acid; 6.3 g boric acid; and 1 M sodium hydroxide (pH D 6.5). The solution was then incubated at 27 ºC for 1 hr. before terminating the reaction by adding 5 mL of 0.5 M NaOH. Blank solutions consisted of 1 mL of pure R2A culture, 1 mL of 50 mM pNPP, 4 mL of MUB, and 5 mL of 0.5 NaOH. Absorbance at 410 nm was read on a Thermo Spectronic 20D and compared to a standard curve generated from pNP (Satta et al., 1979).

Bacterial P solubilization

Phosphorus (P) solubilization is a key trait in bacteria that is routinely measured to assess bacterial strains for plant growth promoting properties. To induce P solubilization activity of bacterial isolates, each isolate was grown in National Botanical Research Institute's phosphate growth medium (NBRIP), which requires bacteria to solubilize calcium phosphate to acquire P for growth (Panhwar, 2012). Briefly, NBRIP consisted of (for 1 L): 10 g glucose, 5 g calcium phosphate, 5 g magnesium chloride hexahydrate, 0.25 g magnesium sulfate heptahydrate, 0.2 g potassium chloride, 0.1 g ammonium sulfate, and 15 g agar (Nautiyal, 1999). P solubilization

was assessed in 96-well plates via a scaled-down version of the United States Geological Survey (USGS) acid-persulfate digestion method (Patton and Kryskalla, 2003). Samples were plated in triplicate, and the average value of those three wells were used as P solubilization for that isolate. Samples were compared to standard curves between 0.005 and 0.1 mg L^{-1} potassium phosphate and read on a Thermo Spectronic 20D at a wavelength of 880 nm.

Preparation for DNA sequencing

The bacterial V4 16S rRNA region was selectively amplified and barcoded using a two-step PCR approach and using established protocols with PNA blockers to prevent plastid and mitochondrial 16S amplification (Lundberg et al., 2013). Barcoded DNA was sequenced on a single lane of an Illumina MiSeq. Forward primers for the first PCR amplification consisted of three 515F universal primers combined with one 515F Crenarchaeota primer and one 515F TM7 primer. Reverse primers were a mixture of three universal 806R primers (**Table 2.1**). Thermal cycler conditions for the primary PCRs for soils were 5 cycles of 95 ºC for 1 min, 50 ºC for 2 min, and 72 ºC for 1 min. A total of 30 mL of the primary PCR products were cleaned with 21 mL of Agencourt AMPure beads and eluted in 21 mL of nuclease-free water. For secondary PCRs, the same reverse and forward primers were used in the 50 mL reaction. Thermal cycler conditions for secondary soil PCRs consisted of denaturation at 95 º C for 45 s followed by 32 cycles of 94 ºC for 15 s, annealing at 60 ºC for 30 s, 72 ºC for 30 s, and final extension at 72 ºC for 30 s.

Sequence analysis

Downstream analysis of sequenced bacterial 16S sequences were conducted in MacQIIME (Caporaso et al., 2010). First, bacterial 16S sequences were assigned to respective samples using barcode sequences designated to each sample prior to amplification and sequencing. Sequences were then clustered into OTUs using an open reference OTU picking protocol using UCLUST at a threshold of 97% similarity (Edgar, 2010). Representative sequences were chosen for each OTU and were identified using the Greengenes database. The lowest amount of sequences per sample was 4,363 and the highest was 25,234 with a mean of 12,593. The sampling depth of 7,497 was chosen to include as many samples as possible without compromising diversity analyses with low sampling depths (**Figure 2.1; all tables and figures are in the Appendix**). Therefore, samples containing less than 7,497 sequences were removed, which eliminated one *P. montana sample* from Ridge (4,363), one *P. montan*a sample from

Valley (5,250), and one *D. excelsa* from Valley (6,895). Finally, both mitochondrial and chloroplast sequences were removed. The relative abundances of all the phyla (**Figure 2.4**) are based on the percentage of 16S rRNA sequences assigned to each phylum.

Statistical analysis

Root phosphatase data were rank transformed prior to performing two-way repeated measures multivariate analysis of variance (MANOVA). Two-way MANOVA avoids errors associated with running multiple ANOVAs because this method can test both enzymes with respect to tree species and site at the same time. The dependent variables were PME and PDE activities, while the independent variables were tree species and site. For each enzyme, three technical replicates of fine root samples were assayed for three individuals of each tree species at each site. An a $\alpha = 0.05$ was chosen to denote statistical significance of either tree species or site on phosphatase activity. Tukey's HSD was used to further examine significant differences of phosphatase activity among samples.

To understand how bacterial community composition responded to tree species and site, a weighted UniFrac distance matrix and principal coordinate analysis plot was constructed using the packages 'phyloseq' and 'vegan' in R (McMurdie and Holmes, 2013; Oksanen et al., 2015). A relative abundance plot was created to show the abundance of phyla in each sample. Principal coordinated analysis (PCoA) was used to visualize the differences between bacterial communities of *P. montana* and *D. excelsa*, where 95% confidence ellipses were drawn around sample points to indicate similarity. Permutational MANOVA (PERMANOVA) was used to statistically test whether bacterial community composition was significantly different between Ridge and Valley and between tree species. An a $\alpha = 0.05$ was also chosen to indicate significance of either site or tree species in determining bacterial community composition.

Results

Resin P availability was lowest in Icacos and highest in Valley

Resin P availability increased from Icacos to Ridge to Valley (**Figure 2.2**) and varied significantly by site (p-value < 0.05) (**Table 2.2**). Post hoc analysis by Tukey's HSD indicated that Icacos and Valley differed significantly in resin P availability. Ridge, which was the intermediate site, did not statistically differ from either Icacos or Valley.

Root phosphatase activity varies with tree species and P availability

Both PME and PDE were significantly different among tree species (Pillai's P = 0.59; p-value < 0.05), though not by site (p-value = 0.70) at an $\alpha = 0.05$ (**Figures 2.3 A, B and Table 2.3**). Furthermore, there was no significant interaction between factors site and tree species. Tukey's HSD of the two-way repeated measures MANOVA indicated that PME and PDE of *P. Montana* differed from all other tree species, except for *M. Garcinifolia*. The average PME in Icacos was 60.03 ± 15.17 µmol pNP $g_{root}^{-1}hr^{-1}$, 36.54 ± 7.56 µmol pNP $g_{root}^{-1}hr^{-1}$ in Ridge, and 24.98 ± 7.88 $g_{root}^{-1}hr^{-1}$ µmol pNP in Valley. For all tree species, PDE activity was roughly one-tenth of PME. Similar to PME, PDE is highest in Icacos (3.63 ± 6.78 µmol bis-pNP $g_{root}^{-1}hr^{-1}$), lower in Ridge (1.31 ± 1.76 µmol bis-pNP $g_{root}^{-1}hr^{-1}$, with the lowest PDE occurring in Valley (1.06 ± 1.37 µmol bis-pNP $g_{root}^{-1}hr^{-1}$). Differences in tree species means, as denoted by the letters in **Figures 2.3 A, B** for PME and PDE indicated that *P. montana* was significantly different between *C. racemiflora, D. excelsa*, and *M. bidentata*. In addition, PDE activity of *P. montana* differed from *C. schreberiana* (p-value < 0.05).

The initial MANOVA of root phosphatase as related to site and tree species indicated the significant effect of tree species but not of site in determining enzyme activity. However, a second MANOVA performed by replacing site with resin P availability showed that resin P availability also influenced enzyme activity(Pillai's P = 0.0901; p-value = 0.0384) (**Table 2.3**), though this is due to a change in degrees of freedom. Resin P availability within each site was significant in determining root phosphatase in contrast to site itself which was not. Indeed, average root PME and PDE with respect to average resin P availability exhibited negative correlations (PME $r^2 = 0.971$; PDE $r^2 = 0.747$) though neither was significant (PME p-value = 0.109; PDE p-value = 0.335) (**Figures 2.3 C, D**). In summary, root phosphatase activity is significantly controlled by tree species, not site, though analysis of resin P suggests the importance of soil P availability in co-influencing enzyme activity in the rhizosphere.

Bacterial community composition in Ridge and Valley was influenced by site

Our analysis of the rhizosphere bacterial community in *P. montana* and *D. excelsa* in Ridge and Valley identified 415 OTUs representing 36 bacterial phyla. The sequence data are stored in NCBI BioProject Accession No. PRJNA412374. Over 50% of the community in both Ridge and Valley came from two phyla: *Proteobacteria* and *Acidobacteria* with no large differences in composition between Ridge and Valley at the phyla level (**Figure 2.4 A**). The

proportion of *Proteobacteria* ranged from 29 to 43% in Valley, whereas it was 37 to 45% in Ridge. The proportion of *Acidobacteria* varied more widely from 13 to 45% in Valley and 19 to 34% in Ridge. The relative abundance plot of bacterial phyla associated with the fine roots of *D. excelsa* and *P. montana* in Ridge and Valley shows no visible differences in relative abundance except for the presence of the *Nitrospirae* phylum found exclusively in Valley.

A weighted UniFrac distance matrix was used to create a PCoA plot with 95% confidence intervals to visually determine similarities among *P. montana* and *D. excelsa* bacterial communities. Points in the PCoA represent each bacterial community, with distances between points indicating similarity or dissimilarity. The resulting plot suggests that bacterial communities are more similar between sites rather than tree species (**Figure 2.4 B**). PERMANOVA using bacterial community composition was used to statistically test whether tree species or site influenced bacterial community composition. At an $\alpha = 0.05$, PERMANOVA results verify that bacterial community composition is influenced by site, not tree species (p-value < 0.05)(**Table 2.5**).

Bacterial isolates from D. excelsa had higher PME and P solubilization activity than isolates from P. montana

A total of 95 isolates from both Ridge and Valley were tested for PME and P solubilization activity after growing the isolates overnight in a P-deficient media (NBRIP). PME activity was found in 36 isolates with a mean of 0.106 ± 0.021 µmol pNP ml^{-1} in isolates from the rhizosphere of *D. excelsa* and a mean of 0.086 ± 0.005 µmol pNP ml^{-1} from *P. montana*. P solubilization occurred in 28 isolates with higher activity in *D. excelsa* (mean = 6.85 ± 21.58 mg P ml^{-1}) relative to *P. montana* (mean = 1.31 ± 1.34 mg P ml^{-1}). A majority (67%) of the isolate library was capable of directly enhancing P availability through either PME or P solubilization activity. However, only 32% of the isolate library showed both PME and P solubilization given our assay conditions . Of the isolates with both PME and P solubilization, 13 isolates were from Ridge, and nine isolates from Valley. Two-way repeated measures MANOVA conducted on PME and P solubilization activity indicated that tree species was a significant factor in determining both in bacterial isolates (p-value < 0.05) but site was not (p-value > 0.05) (**Table 2.6**). Tukey's HSD indicated a significant difference between *P. montana* and *D. excelsa* in PME (p-value = 0.0006), but not P solubilization (p-value = 0.66).

Discussion

A major challenge of integrating root traits and function is capturing their complex interactions with microbial soil communities. However, it is precisely these interactions between roots and root-associated microbes that direct multiple root functions. Despite their importance, the knowledge of these microbial communities relative to specific root traits is still limited (Bardgett et al., 2014). Roots and root-associated microbes actively release phosphatase to enhance P availability, suggesting that both root and microbial functions must be studied in tandem to fully understand these important mechanisms that contribute to tree growth in P limited tropical forests. Recent simulations indicate that phosphatase activity could be an important factor in regulating the size of the carbon sink given that P acquisition may be a limiting factor in continued growth under changing climates (Yang et al., 2016).

Decades of work have established the importance of the soil environment on determining root and bacterial function (Vitousek and Sanford, 1984; Treseder and Vitousek, 2001; Nannipieri et al., 2011; Spohn et al., 2015), which influenced our choice to study root and bacterial phosphatase activity and bacterial community composition at a collection of sites that increased in P availability from Icacos to Ridge to Valley (Mage and Porder, 2013). Our results showed the same pattern and confirmed the strong influence of site on soil P availability, though the only significant difference in resin P availability was between Icacos and Valley. Our first hypothesis was that phosphatase activity would differ based on soil P availability. Two-way repeated measures MANOVA of root and bacterial phosphatase indicated a significant influence of tree species, though not of site. Replacing site with resin P in a MANOVA, however, did suggest that both tree species and resin P availability influenced phosphatase though this is attributable to a change in the degrees of freedom. Linear regressions of average root phosphomonoesterase (PME) and root phosphodiesterase (PDE) at each site with respect to average resin P availability indicated a negatively correlated trend between root phosphatase and P availability. Lastly, average bacterial PME was higher in isolates from the Ridge than those in the Valley suggesting that site and resin P availability are modulating phosphatase in roots and bacteria. As expected, root phosphatase was negatively correlated with resin P availability, supporting the view that root phosphatase may be an important functional trait that forms a critical aspect of nutrient acquisition in low P environments.

Our second hypothesis that phosphatase activity would be influenced by tree species was

supported by both root and bacterial two-way repeated measures MANOVAs albeit our limited sample size. Root PME and PDE activities were significantly different between tree species, most notably between *P. montana* samples and four of the five tree species. Similarly, bacterial PME and P solubilization were influenced by host tree species. Post hoc analysis placed *P. montana* samples as a group separate from the other tree species perhaps because *P. montana* was the only monocot. Monocots were shown to differ from dicots in attributes such as more fibrous root systems (Smith and De Smet, 2012), differences in N:P ratios (Han et al., 2004), and differences in mycorrhizal relationships (Cornwell et al., 2001), which may strongly influence functions in the ecosystem. These results are certainly consistent with previous findings that point to the importance of tree species and of plant functional groups in determining phosphatase activity (Hodge, 2004; White and Hammond, 2008; Lambers et al., 2009; Weintraub, 2011; Keller et al., 2013).

The importance of tree species on root phosphatase can also be tied to the combination of conservative and acquisitive traits that regulate tissue P demand and therefore the necessity of acquiring P via root phosphatase. Plants can respond to P limitation by altering functional traits to either increase P efficiency or P acquisition, though it is likely that different tree species use varying combinations of both to adjust to local nutrient supplies (White and Hammond, 2008). Within the tropics, multiple lines of evidence confirm that trees are capable of efficiently using P through high P resorption, P recycling, and reduction of P concentrations in metabolic nucleic acid compounds (Vitousek and Sanford, 1984; Hidaka and Kitayama, 2011). These different mechanisms result in a variety of tissue P requirements for different tree species and therefore different responses to P availability and need for root phosphatase (Ushio et al., 2010; Keller et al., 2013). The lack of an interaction term between site and tree species suggests that tree species is driving differences in root phosphatase activity independently. However, it is important to note that site is likely to be a confounding factor with tree species given that the soil environment can modify these functional traits (Lambers et al., 2006; Richardson et al., 2009; Niu et al., 2013).

Although our alpha level of 0.05 excluded site as a significant determinant of root and bacterial phosphatase activity, we suspect that they are likely influenced by feedbacks between site and tree species. P availability and tree species have consistently been found to regulate root and bacterial function (Treseder and Vitousek, 2001; Costa et al., 2006; Lambers et al., 2006; Haichar et al., 2008; Bardgett et al., 2014; Hinsinger et al., 2015). For example, differences in

phosphatase activity between root samples of the same tree support the notion that heterogeneous distribution of P within each site can cause variation in measured activity. Since roots must respond to local conditions, microsite variations in nutrient content, microbes, and competing roots likely modify phosphatase activity (Baldrian, 2014). Alternatively, differences in phosphatase activity between root samples of the same individuals and of tree species could be due to variation in the root orders collected and ensuing differences in the microbes attached to root tissue.

Root order was shown to correspond to different roles in the root system where the first and second orders, or the most distal roots, are associated with higher rates of exudation and uptake than those of higher orders typically associated with transport (McCormack et al., 2015). As such, samples with a greater proportion of higher order roots may have shown much less phosphatase activity than samples of the same individual made up of predominantly first- and second-order, absorptive roots. Preliminary observations of *D. excelsa* and *P. Montana* roots show that the branching intensity (number of first-order roots per centimeter of second-order roots) of *D. excelsa* (5.58) was higher compared to *P. montana* (2.70) (Daniela Yaffar, personal communication). The difference in branching intensity between *D. excelsa* and *P. montana* may in part explain why phosphatase activity is higher in *D. excelsa* as it has a much higher amount of first-order (resource acquisitive) roots. Similarly, a comparison between lianas and trees demonstrated that roots of lianas seem to favor high P acquisition, correlated with root traits such as greater root branching intensity, higher N and P concentrations, and higher specific root lengths (Collins et al., 2016). Future work will involve examining how root morphology may impact phosphatase activity and whether the release of phosphatase changes depending on the effectiveness of root architectural and morphological traits to find patches of orthophosphate. Indeed, the role of phosphatase activity in determining P mineralization is likely to depend on merging how phosphatase correlates with other root functional traits and the microbial community.

Finally, our third hypothesis that bacterial communities would be largely influenced by tree species was contradicted by results that indicated the significance of site, but not of tree species in determining bacterial community composition. In both Ridge and Valley bacterial communities, the major phyla representing over half of the microbial communities were *Proteobacteria* and *Acidobacteria*, both of which tend to be common in soils (Bulgarelli et al.,

2013). However, the phylum *Nitrospirae*, known to be the most diverse group of nitrite-oxidizing bacteria (NOB), only occurred in the Valley (Daims et al., 2015). Conditions in Valley likely favor *Nitrospirae* because of much wetter conditions that create an anoxic soil environment. While there is no question that soil conditions impact the composition of bacterial communities in significant ways through soil pH (Fierer and Jackson, 2006) and microscale heterogeneity (Vos et al., 2013), the intimate association between plants and their root bacterial symbionts certainly affirms the importance of tree species as key factor (Carney and Matson, 2006; Ushio et al., 2008, 2010; Bulgarelli et al., 2013; Philippot et al., 2013). One theory of how rhizosphere microbial communities are formed suggests that soil conditions, such as nutrient availability, initially determine soil microbial community composition. Growing plants then release root exudates which further alter the soil environment, fostering a unique microbial community distinct from bulk soil (Bulgarelli et al., 2013). This two-step model may explain why microbial community composition is more strongly influenced by soil conditions, though the limited sampling size may have obscured stronger effects of tree species on bacterial composition. Interestingly, although bacterial community composition was influenced by site, a two-way repeated measures MANOVA of bacterial PME and P solubilization indicated that these two functions were only influenced by tree species and not site.

PME activity and P solubilization of bacterial isolates were higher in isolates from *D. excelsa* than those from *P. montana*. However, PME was more prevalent in the bacterial isolates collected, despite the importance of P solubilization in enhancing P availability. This might be due to the limitations of the P solubilization assay and the differences in target compounds. First, P solubilization is typically measured using calcium phosphate as a P source, requiring bacterial colonies to solubilize calcium phosphate to survive. However, this favors only those bacterial strains capable of solubilizing calcium phosphate, limiting extrapolation of this function to the broader microbial community. However, P solubilization is a commonly used and relied upon criteria to screen for plant growth promoting activities, thus why we measured it here. Second, phosphatase and P solubilization act on different compounds in the soil. Phosphatase enzymes mineralize organic compounds, which form an abundant portion of total P in tropical forests (Turner and Engelbrecht, 2011). In contrast, P solubilization targets phosphate bound to inorganic compounds, which is a much smaller and more recalcitrant pool (Saghir et al., 2014). It is possible that substrate abundance for PME encourages more members of the bacterial

community to express this function. Furthermore, among the 95 isolates tested, only a few isolates were capable of both PME and P solubilization activities, suggesting that it may be uncommon to possess both functions in the larger bacterial community. Given the paradigm that less than 1% of the microbial community can be cultured, our bacterial isolates likely do not capture the full functional potential of the broader microbial community. However, our bacterial isolates provide an opportunity to test bacteria and root interactions in controlled greenhouse settings. Furthermore, our results indicated that tree species played an important role in determining root and bacterial PME activity, supporting efforts to build more species-specific trait understanding of P mineralization.

The root system is highly plastic, responding to variations in P availability through changes in root morphology, architecture, exudation, and interactions with soil microbes (Lambers et al., 2006; Niu et al., 2013). In addition to root and bacterial phosphatase and P solubilization, there are multiple mechanisms critical to understanding how the root system adjusts to P limitation. For example, P deficiency can induce an increase in lateral roots near the soil surface and morphological changes such as increasing root length, root turnover, and higher biomass allocation to roots (Lynch and Brown, 2001). Furthermore, mycorrhizal hyphae are a critical pathway for P uptake because fungal hyphae are much thinner than the smallest roots, enabling a much larger surface area with which to absorb orthophosphate and enhance foraging of soil P sources (Plassard and Dell, 2010; Smith and Smith, 2011). Numerous reviews describe how this intimate association between trees and fungi influence root branching, fine roots, root: shoot ratio, specific root length, and responses to elevated CO_2 as well as the finer details of the root–fungal interface and the exchange of nutrients (Bucher, 2007; Plassard and Dell, 2010; Smith et al., 2011; Bardgett et al., 2014; Liu et al., 2015). However, our results address the (much less known) bacterial component of the plant microbiome.

Root and bacterial phosphatase activity are central components in plant P acquisition and a source of major uncertainty in understanding P mineralization, forest productivity, and modeling tropical forest growth (Cernusak et al., 2013; Hofhansl et al., 2016). The variation of root phosphatase with tree species is encouraging as it may be an important trait for predicting how different tree species respond in altered climatic regimes. Although tropical forests are immensely diverse, our results with our tree species begin pairing root function with bacterial function and community composition to improve understanding of one critical aspect of P

dynamics in tropical forests – phosphatase activity. Our main result was that root and bacterial phosphatase, though not bacterial community composition and P solubilization, vary with tree species and soil P availability. Average values of root phosphatase activity show a promising negative correlation with resin P availability that encourages future empirical and modeling research aimed at building trait-based understanding of P dynamics in tropical forests.

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Appendix

Table 2.1 List of primers used for bacterial community PCR amplification

Sequence Description - Forward	Sequence	Scale
515F_f1	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNNNNNGAGTGCCAGCMGCCGCGGTAA	20 nm Ultramer
515F_f2	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNTNNNNGAGTGCCAGCMGCCGCGGTAA	20 nm Ultramer
515F_f3	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNCTNNNNGAGTGCCAGCMGCCGCGGTAA	20 nm Ultramer
515F_f4	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNACTNNNNGAGTGCCAGCMGCCGCGGTAA	20 nm Ultramer
515F_f5	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNGACTNNNNGAGTGCCAGCMGCCGCGGTAA	20 nm Ultramer
515F_f6	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNTGACTNNNNGAGTGCCAGCMGCCGCGGTAA	20 nm Ultramer
515F_f1C	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNNNNNGAGTGCCAGCMGCWGCGGTAA	20 nm Ultramer
515F_f1TM7	GCCTCCCTCGCGCCATCAGAGATGTGTATAAGAGACAGNNNNNNNNGAGTGCCAGCMGCCGCGGTCA	20 nm Ultramer
Sequence Description - Reverse	**Sequence**	**Scale**
806R_f1	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNACGGACTACHVGGGTWTCTAAT	20 nm Ultramer
806R_f2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNTNNNACGGACTACHVGGGTWTCTAAT	20 nm Ultramer
806R_f3	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNCTNNNACGGACTACHVGGGTWTCTAAT	20 nm Ultramer
806R_f4	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNACTNNNACGGACTACHVGGGTWTCTAAT	20 nm Ultramer
806R_f5	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNGACTNNNACGGACTACHVGGGTWTCTAAT	20 nm Ultramer
806R_f6	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNTGACTNNNACGGACTACHVGGGTWTCTAAT	20 nm Ultramer

Table 2.2 ANOVA of soil resin P across all three sites

Resin P ANOVA table

	Degrees of freedom	Sum of squares	Mean squares	F-value	Pr (>F)
Site	2	0.7643	0.3822	4.9789	0.0155*
Residuals	24	1.8421	0.0768		

* Denotes p-value < 0.05.

Table 2.3 Site and tree species two-way repeated measures MANOVA of root PME and PDE activities

Site × tree species root phosphatase

	Degrees of freedom	Pillai	Approximate F	Num. degrees of freedom	Den. degrees of freedom	Pr (>F)
Site	2	0.1208	2.2195	4	138	0.0700
Species	5	0.5798	5.6349	10	138	<0.001*
Site:Species	2	0.0414	0.7306	4	138	0.5725
Residuals	69					

* Denotes p-value < 0.05.

Table 2.4 Resin P and tree species two-way repeated measures MANOVA of root PME and PDE

Resin P × tree species root phosphatase

	Degrees of freedom	Pillai	Approximate F	Num. degrees of freedom	Den. degrees of freedom	Pr (>F)
Resin P	2	0.0901	3.4180	2	69	0.0384*
Species	5	0.5946	5.9232	10	140	<0.001*
Resin P:Species	2	0.0357	0.6369	4	140	0.6370
Residuals	69					

*Denotes p-value < 0.05.

Table 2.5 PERMANOVA for analysis of similarity/dissimilarity among bacterial community from *D. excelsa* and *P. montana.*

Bacterial PME and PDE

	Degrees of freedom	F-value	Pr (>F)
Site	1	2.5658	0.014*
Species	1	0.7345	0.642
Permutations	999		

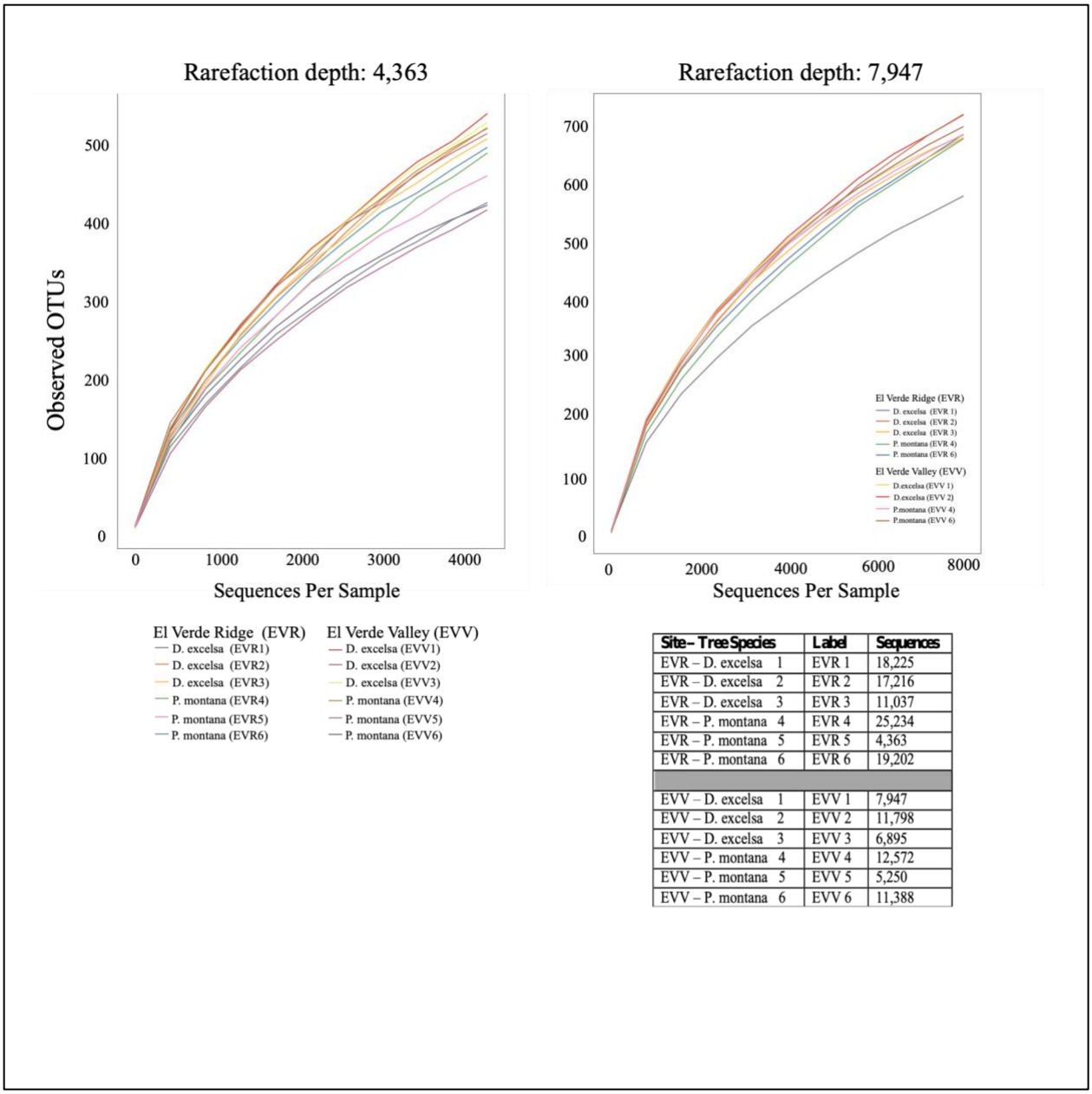
*Denotes p-value < 0.05.

Table 2.6 Two-way repeated measures MANOVA of bacterial PME and P solubilization activity.

Bacterial PME and P solubilization MANOVA

	Degrees of freedom	Pillai	Approximate F	Num. degrees of freedom	Den. degrees of freedom	Pr (>F)
Site	1	0.2057	2.9792	2	23	0.0707
Species	1	0.3868	7.2540	2	23	0.0036*
Site:Species	1	0.1416	1.8977	2	23	0.5725
Residuals	24					

*Denotes p-value < 0.05.



Site– Tree Species	Label	Sequences
EVR – D. excelsa 1	EVR 1	18,225
EVR – D. excelsa 2	EVR 2	17,216
EVR – D. excelsa 3	EVR 3	11,037
EVR – P. montana 4	EVR 4	25,234
EVR – P. montana 5	EVR 5	4,363
EVR – P. montana 6	EVR 6	19,202
EVV – D. excelsa 1	EVV 1	7,947
EVV – D. excelsa 2	EVV 2	11,798
EVV – D. excelsa 3	EVV 3	6,895
EVV – P. montana 4	EVV 4	12,572
EVV – P. montana 5	EVV 5	5,250
EVV – P. montana 6	EVV 6	11,388

Figure 2.1 Rarefaction curves for bacterial community sequencing at depth = 4,363 and depth = 7, 497

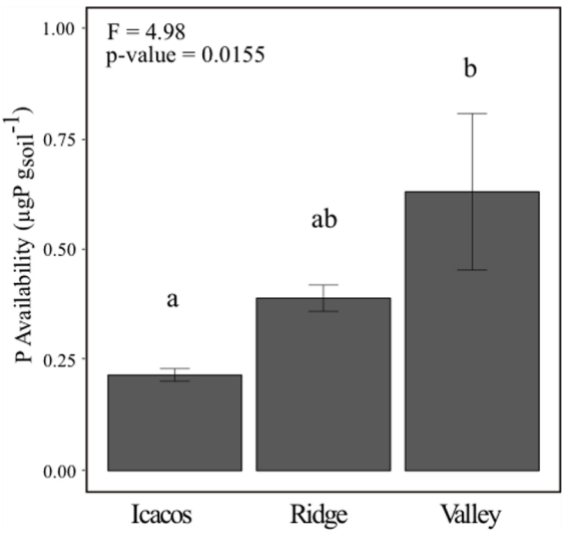


Figure 2.2 Soil resin P availability across all three sites with letters denoting similarity or dissimilarity of group means. Error bars represent mean ± standard error of the mean of Icacos (n = 10), Ridge (n = 9), and Valley (n =9)

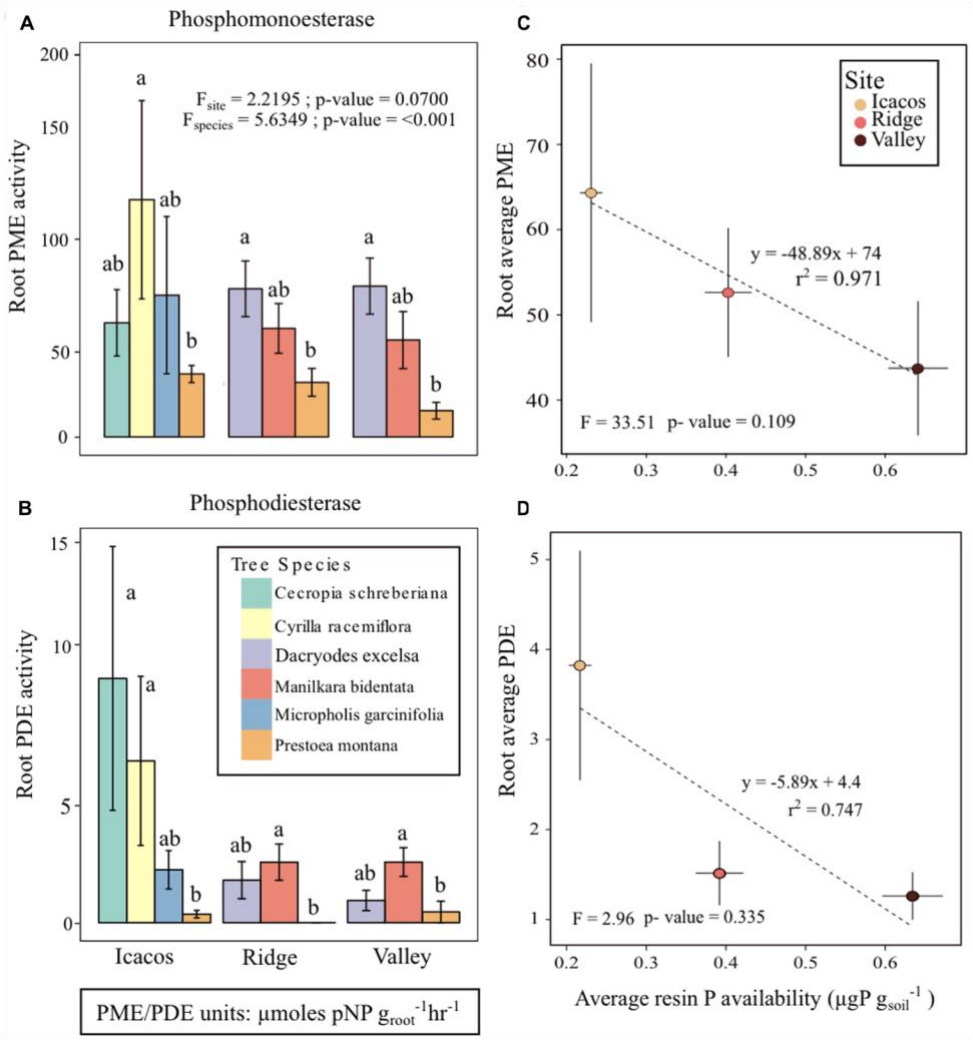


Figure 2.3 Root phosphomonoesterase (PME) activity (A), root phosphodiesterase (PDE) activity (B), average root PME activity plotted with average resin P availability at each site (C), and average root PDE activity with average resin P availability (D). Letters represent differences in group means among tree species and error bars are mean SE with n = 3 individual trees per species at each site.

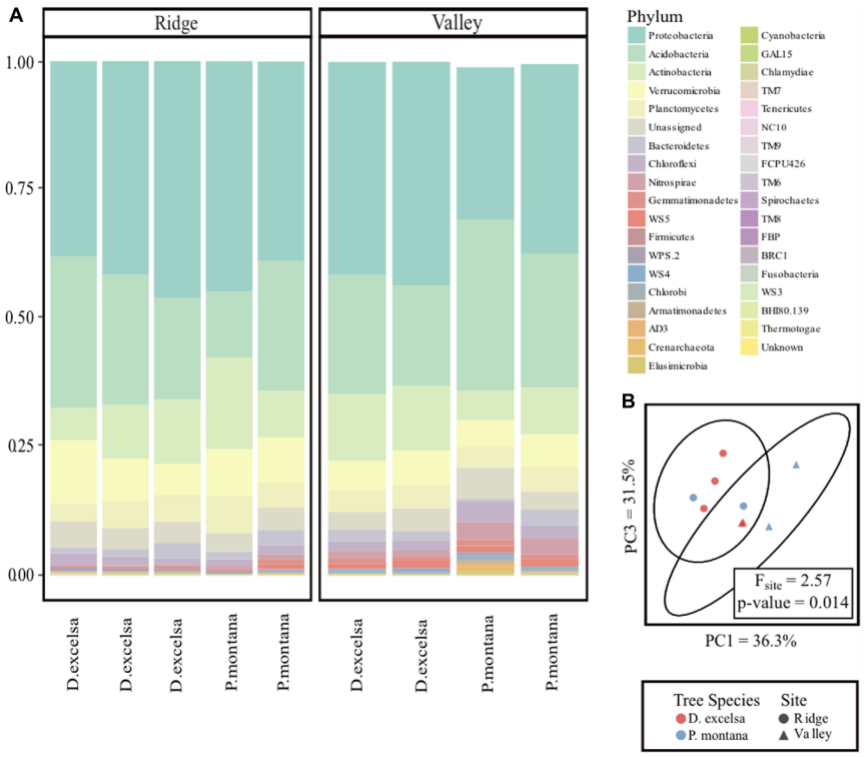


Figure 2.4 Comparison of bacterial communities between Ridge and Valley sites and *D. excelsa* and *P. montana*. Relative abundance plot of bacterial phyla > 2% of the community (A). One *P. montana* sample in the Valley contained multiple phyla each representing < 2% of the community. These were excluded from the plot resulting in a shorter bar. PCoA with ellipsoids representing 95% confidence intervals (B).

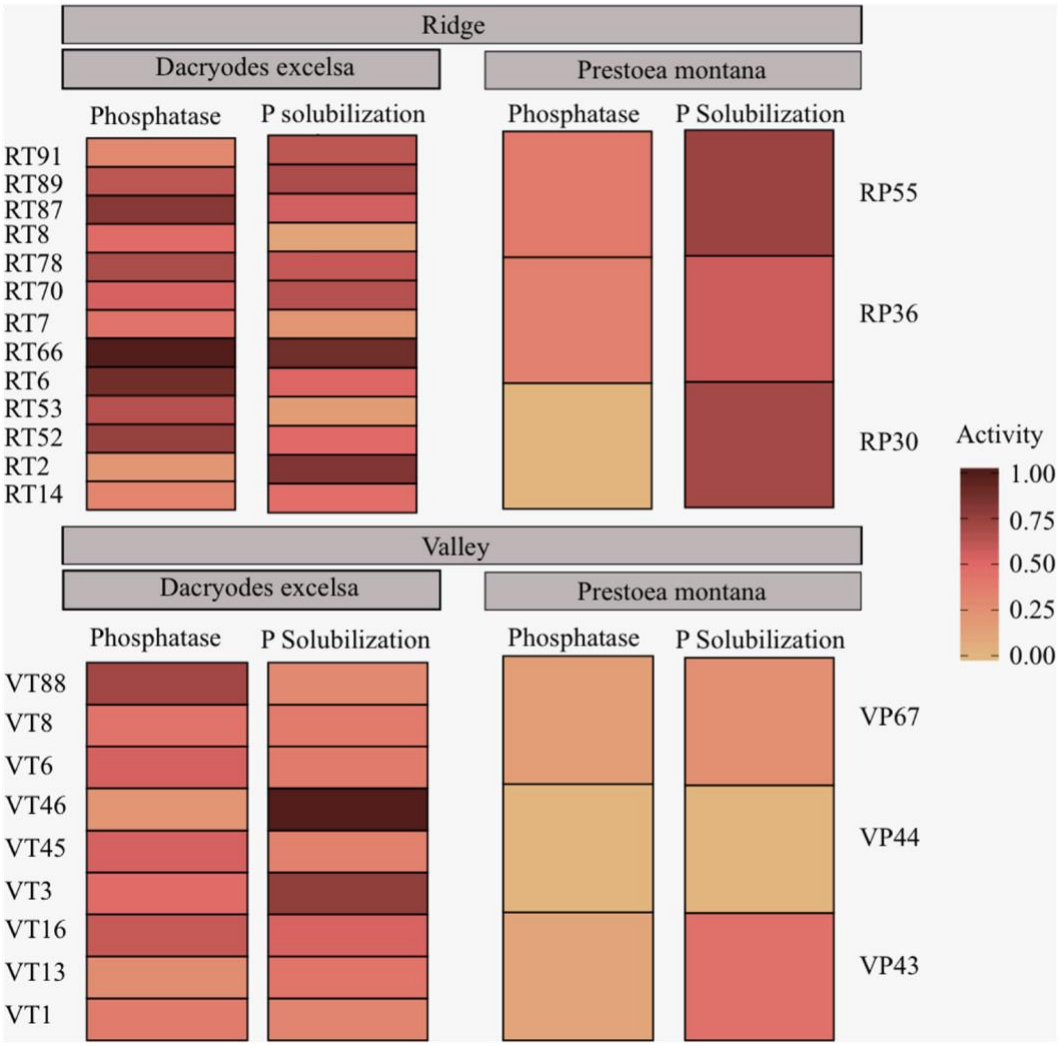


Figure 2.5 Functional assays of phosphatase activity and P solubilization activity of bacterial isolates from the rhizosphere. Ridge isolates are displayed on top and Valley isolates are on the bottom. Isolates from *D. excelsa* are on the left and isolates from *P. montana* are on the right.

Chapter 3

Bringing function to structure: Root-soil interactions shaping phosphorus availability through a soil profile in Puerto Rico

My use of "we" in this chapter refers to my co-authors and myself. This chapter is a previous iteration of the submitted manuscript of the same title, authored by Kristine Grace M. Cabugao, Daniela Yaffar, Nathan Stenson, Joanne Childs, Jana Phillips, Melanie A. Mayes, Xiaojuan Yang, David J. Weston, and Richard J. Norby *submitted* to *Functional Ecology* in April 2020.

Abstract

Large areas of highly productive tropical forests occur on weathered soils with low concentrations of available P. Mineralization of organic P phosphomonoesters by acid phosphomonoesterase (PME) enzymes released from roots and microbes is thought to contribute to plant uptake by increasing P availability within these soils. However, there are many uncertainties concerning how root and soil factors regulate PME. We aimed to address how PME from the root surface (root PME) and PME produced by microbes in the soil (microbial PME) changed throughout soil depth and which root and soil factors were most predictive of PME activity. We measured microbial PME down to 1 m, root PME to 30 cm and collected data on fine-root mass density, specific root length, soil P, bulk density, and soil texture. We found that soil PME decreased with soil depth, but that root PME only declines with depth when root PME is expressed per soil volume. Soil PME increased with fine-root mass density and total P, which together explained over 50% of the variation in soil PME. Meanwhile, over 80% of the variation in root PME was attributed to specific root length (positive correlation) and resin P (negative correlation). Our results indicate that a both fine-root traits and soil P data are necessary to fully understand the role of PME in organic P mineralization. Furthermore, our data can better improve estimates of the biochemical mineralization parameter in ecosystem models with existing root distribution and soil depth structures.

Introduction

Tropical forests disproportionately influence the global carbon cycle, exchanging the largest amount of carbon between atmosphere and terrestrial ecosystems while also storing an estimated 25% of global terrestrial plant biomass and about one third of soil carbon (Jobbágy & Jackson, 2000; Lewis et al., 2009; Yang, et al., 2014). Despite their importance, the mechanisms that regulate tropical forest productivity remain poorly understood. In many natural ecosystems, nutrient availability, particularly of nitrogen and phosphorus (P) commonly limits productivity (Dalling et al.2016). Concentrations of P in the form readily absorbed by plants – orthophosphate – are significantly lower in tropical regions relative to temperate ecosystems, yet every year tropical forests alone account for one third of global NPP (Reed et al., 2011; Dalling et al., 2016). Root traits and functions, like phosphatase activity, that increase P availability are increasingly recognized as a vital component of understanding and modeling how tropical trees support such high levels of productivity despite the vast tracts of P-limited soils underneath many tropical forests (Norby, 2011; Warren et al., 2015; Wieder et al., 2015; Achat et al., 2016).

Phosphorus is absorbed by roots in the form of orthophosphate ions ($H_2PO_4^-$ or HPO_4^{2-}) and is an essential nutrient for photosynthesis, nucleic acid synthesis, and energy transfer (Vitousek and Sanford, 1984; Vitousek et al., 2010; Raven, 2015; Dalling et al., 2016). However, orthophosphate generally comprises the smallest proportion of total P in most tropical soils due to prolonged weathering and strong reactions with soil minerals that remove it out of soil solution (Brenner et al. 2018). Phosphorus enters the soil through weathering of the underlying rock, meaning that younger ecosystems typically have higher concentrations of available P (orthophosphate in soil solution and orthophosphate loosely bound to mineral surfaces). However, as soil development and weathering progress, the proportion of available P decreases due to leaching and reactions that sequester orthophosphate into organic or inorganic P forms. Inorganic P in forms that are considered biologically inaccessible is considered unavailable or occluded P (Walker and Syers, 1976). Many humid tropical forests are old ecosystems that have experienced thousands to millions of years of weathering without any disturbances that might expose new rock material to replenish soil P resulting in low concentrations of available P (Dalling et al., 2016; Reed et al., 2011). A global map of various forms of P as measured using Hedley fractionation confirmed that the proportion of available P (~ 20%) is much lower than that of unavailable P (~ 50-59%) in Oxisol and Ultisol soils that occur under a majority of

tropical forests (Yang & Post, 2011). However, an average of 26% of total soil P occurs as organic P, which roots and soil microbes mineralize into orthophosphate ions using phosphatase enzymes (Tarafdar & Claassen, 1988; Turner & Engelbrecht, 2011). Thus, P acquisition in tropical soils likely involves both maximizing capture of available P and accessing the soil organic P pool using phosphatase enzymes, both of which are influenced by root traits and function.

Root traits are defined as characteristics that influence plant growth, survival, and fecundity (Violle et al., 2007; McCormack et al., 2017). Understanding how fine-root traits mediate the capture of available inorganic P and mineralization of soil organic P is crucial to connecting the soil P cycle to productivity in tropical forests on P-limited soils. Increasing P acquisition is associated with specific changes in the morphology, physiology, architecture, and microbial associations of fine roots (Lambers, 2006). For example, specific fine-root length and surface area increase in low P sites (Treseder & Vitousek, 2001; Santiago, 2015; Ushio et al., 2015), and a majority of tropical trees form symbiotic interactions with arbuscular mycorrhizal fungi which also increase the soil volume explored (Reed et al., 2011). While these traits maximize capture of available inorganic P, the release of phosphatase enzymes from the root surface enables P acquisition from the much larger soil organic P pool. These enzymes, exuded into the soil by both roots and microbes, are widely regarded as an important mechanism to increase P availability by breaking down soil organic P (McGill & Cole, 1981; Richardson et al., 2009; Tarafdar & Claassen, 1988; Yang & Post, 2011).

Extracellular phosphatase enzymes (EC 3.1.3) increase P availability by hydrolyzing phosphoester bonds, essentially recycling organic P into inorganic available P (Helal, 1990). In tropical soils, an estimated 68% - 96% of soil organic P occurs as phosphomonoester compounds such as inositol phosphates, sugar phosphates, and mononucleotides (Turner & Haygarth, 2005; George et al., 2006; Nannipieri et al., 2011; Turner & Engelbrecht, 2011). Phosphomonoesters in acidic tropical soils are mineralized by acid phosphomonoesterase (PME; EC 3.1.3.2)(Juma & Tabatabai, 1978; Nannipieri et al., 2011). The prevalence of phosphomonoesters in organic P pools coupled with the large proportion of unavailable inorganic P suggests that PME activity may be an important root function to increase P availability in tropical forests (Richardson et al., 2004; George et al., 2017). Furthermore, P uptake studies using 32P demonstrated that P uptake is positively correlated with root PME (Lee, 1988), and that mineralization of organic P by

phosphatase enzymes close to the root surface was responsible for increasing P uptake (Tarafdar & Claassen, 1988; Tarafdar & Jungk, 1987). Thus, PME influences both the shift between unavailable and available P as well as increasing P availability within the rhizosphere where plant uptake occurs.

Incorporating the links between root traits, P availability, and plant growth is a necessary step in reducing model uncertainty of organic P mineralization throughout soil depth, particularly in tropical forests where productivity is considered sensitive to available P (Reed et al., 2015). Model simulations indicate that soil organic P mineralization was overestimated in Oxisols because estimates of biochemical mineralization – phosphatase – were poorly constrained (Wang et al., 2010). Ecosystem models already incorporate soil layers and some include root depth distribution (Koven et al., 2013; Warren et al., 2015). However, biochemical mineralization is currently modeled as a function of supply and demand of available P, irrespective of root traits involved that contribute to influencing supply (i.e. phosphatase) and demand (Goll et al., 2012). Recent studies have already begun to link PME activity to root morphology and anatomy, demonstrating that root PME was associated with increasing specific root length (Kitayama, 2013; Lugli et al., 2019), increasing specific root surface area, and decreasing root tissue density (Ushio et al., 2015b). Our overarching goal was to better understand root and soil PME within the context of root traits and soil P, essentially bringing function – biochemical mineralization – to the existing soil and root structure incorporated into ecosystem models.

Phosphomonoesterase is considered an adaptive enzyme such that levels of activity correspond to changes in plant demand and P supply (Nannipieri et al., 2011). Thus, both plant and soil factors likely act in concert to control PME activity. However, there is great uncertainty about how PME correlates with changes in root and soil factors throughout depth, which root traits and soil factors influence PME, and whether the same factors are significant to regulating PME in bulk soil (soil PME) or PME specifically exuded onto the root surface (root PME). Therefore, we measured soil PME down to 1 m and root PME to 30 cm in the Luquillo Experimental Forest while taking corresponding measurements of fine-root mass density, specific root length, and a host of soil factors such as resin P (considered available P), organic P, soil texture, and water content.

We expected that both root and soil PME would decrease with depth because of the general decline of biological activity due to decreasing fine-root mass density and resin P

(available P) (Jackson et al., 1997; Nannipieri et al., 2011). In a previous experiment, we found that root PME was negatively correlated with increasing resin P (Cabugao et al., 2017). Furthermore, since root PME is exuded at the root surface, we expected that it would increase with specific root length, consistent with other experiments (Kitayama, 2013; Lugli et al., 2019). However, soil microbes also release phosphatase enzymes independent of root systems in the bulk soil. Thus, while root PME may be more responsive to resin P and specific root length, soil PME may respond more strongly to organic P than root traits.

Previously, we found that root PME differed among tree species and sites with contrasting P availabilities (Cabugao et al., 2017). Our aim was to build on previous work by developing relationships between fine root traits and PME as modified by soil conditions and P availability throughout the soil profile. Furthermore, although ecosystem models include root distribution; they do not currently include associated PME activity. By measuring PME and coupling it with existing root and soil parameters, we can improve our understanding of root mechanisms to access P down the soil profile while also creating a dataset that can be used to evaluate model simulations of the phosphorus cycle in tropical forests.

Materials and Methods

Study sites

We collected soil cores from four sites within the Luquillo Critical Zone: El Verde Ridge (EVR), El Verde Valley (EVV), Icacos Ridge (ICR, and Icacos Valley (ICV). The Luquillo Critical zone is within the Luquillo mountains of northeastern Puerto Rico (18°30'N, 65°80'W) and is comprised of two different types of parent material. The two El Verde sites (EVR and EVV) occur on Oxisol soils, stemming from lower-Cretaceous volcaniclastic parent material. In contrast, the two Icacos sites (ICR and ICV) are on early-Tertiary age quartz-diorite parent material, resulting in Inceptisol soils. Within El Verde, the soil is finely textured, which contrasts with the Inceptisols in Icacos that are much coarser. Soils of both soil orders tend to be strongly acidic and abundant in iron and aluminum hydroxides within the clay fractions (Stone et al., 2014). Dominant vegetation in the Luquillo Critical Zone is influenced heavily by elevation whereby our El Verde sites at low elevation (< 600 meters above sea level) were dominated by *Dacryodes excelsa* Vahl (Tabonuco). At higher elevations (600-800 meters above sea level), such as in our Icacos sites; vegetation was composed primarily of *Cyrilla recemiflora* L. (Palo Colorado). Notably, the only tree species similar across the two sites was *Prestoea montana*

Hook (Sierra Palm) (Brown 1983; Stone 2014). Mean annual temperature decreases with elevation from 24 ºC at 300 m to 21 ºC at 800 m. Precipitation across the same elevation gradient increases with elevation from 3000 mm y^{-1} to 4000 mm y^{-1} (Brown 1983; Stone 2014). In terms of P content, Oxisols on volcaniclastic parent material in El Verde were found to contain double the amount of soil P when compared with Inceptisol soils occurring on quartz-diorite parent material (Mage & Porder, 2013).

Sample collection

In February 2019, we collected two 1-m cores and one 30-cm core at three replicate locations at each site (n = 3). We used a split core (30 cm × 5 cm) to sample every 5 cm down to 1 m for resin P (available P), organic P (portion of total P that includes substrates for phosphomonoesterase), total P, and soil acid phosphomonoesterase (PME) at the following depth increments: 0-5 cm, 7-12 cm, 15-20 cm, 25-30 cm, 45-50 cm, and 80-90 cm. we separated sections of the same 1 m core except for 0-5 cm and measured root traits to ensure enough undisturbed root material for root biomass measurements. Thus, root traits were measured at depths: 5-7 cm, 12-15 cm, 20-25 cm, 30-45 cm, 50-80 cm, and 90-100 cm. Analyses and graphs were based these nominal increments, though the archived dataset maintains the field-noted depths (dataset; Norby 2019). The second 1 m core was sampled for bulk density, soil texture, and soil moisture using a bulk density core sampler (AMS, American Falls, Idaho). The third core was used for paired measurements of root and soil PME, fine-root surface area, and fine root mass density only to 30 cm. We shipped soil depth increments on blue ice overnight for processing at Oak Ridge National Laboratory.

Soil physical characteristics

We extracted soil samples collected for physical characterization from the 5 cm × 5 cm (90.59 cm^3) bulk density core liner and immediately sealed them into moisture-tight sample bags. In the laboratory, we removed stones and occasional large root fragments and determined their volume by water displacement. We measured fresh mass of the soil, and then dried the soil at 105°C for 2 days. We calculated bulk density as dry mass divided by core volume corrected for removed material and calculated soil moisture as the fresh mass minus dry mass divided by dry mass. We determined soil particle size fractions (sand, silt, and clay) using the bouyoucos hydrometer method on oven-dried and ground samples (Gee & Or, 2004).

Resin P

The resin P method, which measures the form of inorganic P (orthophosphate) available to plants and microbes relies on anion exchange resin membrane strips charged to attract phosphate ions, essentially approximating root depletion of P from the soil solution (Calvert et al., 1993). We charged resin strips with sodium bicarbonate (0.5M) to ensure a positive charge prior to placing them in a soil suspension made from ~8 g of fresh soil mixed with 80 mL of deionized H2O. Phosphate ions in the solution adhere to the strips during a 24-hr. incubation on a shaker. Then, we removed phosphate ions adsorbed on the resin strips by shaking resin strips in 50 mL of 0.25 M H_2SO_4 for 1 hr. and quantifying the P concentration using a Lachat QuikChem 8500 method 10-115-01-1-B modified with a 60 cm sample loop (Hach, Loveland, Colorado, USA). We measured GWC concurrently for each sample to express results on a per unit soil dry mass basis. We presented results for resin P at each depth as the average of the three replicate 1 m cores at each site.

Organic P

We measured organic P as the sum of acid and alkali extractions (Bowman, 1989; Condron et al., 1990). In a 50 mL falcon tube, we added 2 g of field-moist soil, 3 mL of 18 M H_2SO_4, and 4 mL of deionized water vortexing frequently to ensure a homogenous slurry. Next we brought up total solution volume to 48 mL using deionized water and centrifuged the samples for 10 mins at 2500 rpm. We filtered the supernatant through Whatman No. 1 paper and saved the filtered solution as the acid extract. We washed the remaining soil thoroughly with deionized water and centrifuged. We put centrifuged soil and filter paper from the acid extraction on a shaker with 98 ml of 0.5 M NaOH for 2 hr. at room temperature. We centrifuged and filtered the samples to get our alkali extracts. Acid and alkali extracts were analyzed using a Lachat QuikChem 8500 method 13-115-01-1-B. We measured gravimetric water content (GWC) concurrently for each sample and presented organic P results per unit dry soil mass as the average of the 1 m cores taken at each of the three replicate locations at each site.

Total soil P

We used 400 mg of field-moist soil from each depth increment at each replicate location within sites to measure total soil P. Samples were ground in 50 mL falcon tubes using a Geno/Grinder 2010 (Spex Sampler Prep, Metuchen, New Jersey, USA). All samples were analyzed for total soil P using a Lachat BD40 block digestor for high temperature digestion and a

Lachat QuikChem 8000 series for flow injection analysis method 13-115-01-1-B (Hach, Loveland, Colorado, USA).

Root biomass and root %P concentration

We manually picked roots from each depth increment of all 1 m core soil samples and separated roots into two categories: ≤ 1 mm in diameter and > 1 mm in diameter. Previous analysis of fine roots from these sites indicated that roots ≤ 1 mm in diameter were generally 1rst and 2nd order roots that had higher N and P concentrations than 3rd order roots (Daniela Yaffar; unpublished data). Typically, 1^{rst} and 2nd order fine roots are classified as "absorptive", referring to their primary function as active sites of water and nutrient uptake. In contrast, roots of higher orders are considered transport fine roots associated with the transfer and storage of nutrients (McCormack et al., 2015). Although the 1 mm diameter threshold for delineating 1rst and 2nd order roots is not true for all ecosystems, we thought this was sufficient in our samples to characterize "absorptive" roots in our samples because this was a general trend in our sites (Daniela Yaffar; unpublished data). We dried roots at 65° C for 2 days and weighed them to obtain root biomass per depth, replicate location, and site. We analyzed fine-root P concentration at a community-level by subsampling 100 mg of root dry mass with the same digestion protocol as total soil P described above.

Specific root surface area

We rehydrated dried root samples used for root phosphomonoesterase assays and applied a linear regression to correct for dehydration (described below) prior to calculating specific root surface area using the equation from WinRhizo (Regent Instruments Inc., Canada). We scanned roots from each depth using WinRhizo (Version 2012B). To correct for differences in root length and root diameter between dried and fresh roots, we first correlated root length and root diameter from root samples measured fresh and then dried (modified from Bergmann et al. 2017). We performed a linear regression with the samples and applied the regression equation to our samples where x denotes dried root values and y denotes expected fresh root values. For length, we found that fresh root length could be determined using the equation: $y = 0.92x-0.48$ ($r^2 = 0.99$) and that fresh root diameter could be found using $y = 1.12x-0.069$ ($r^2 = 0.91$). We estimated root surface area using calculated values of diameter and length from these equations.

Soil and root phosphomonoesterase activity

Within 1 week of sampling, we measured soil and root phosphatase activities using a

modified version of the colorimetric para-nitrophenyl phosphate (pNPP) assay (Tabatabai & Bremner, 1969; Png et al., 2017). We used 1 g of fresh soil in 4 mL of Tris-maleate buffer (for 1 L: 60 g maleic acid, 60 g tris-(hydroxymethyl)-aminomethane; pH = 6.5) with 1 mL 25 mM pNPP (1.855 g pNPP into 200 mL tris-maleate buffer) as a substrate. We incubated soil samples for 1 hr. at 27 °C and terminated the reactions by adding 1 mL of 0.5 M calcium chloride and 0.5 M sodium hydroxide. We took 2 ml aliquots of the terminated solution and centrifuged them at 10,000 rpm for 2 mins. To read the concentration of para-nitrophenol, we took 1 ml of the supernatant and added it to 5 ml of milliQ water. Then, we took 2 ml of the diluted solution in a square cuvette and read absorbance at 410 nm. Bulk soil phosphatase activity comprises both microbially-derived phosphatase and those exuded by roots.

Root phosphatase assays aim to capture the phosphatase activity occurring from exuded phosphatase enzymes at the root surface (rhizoplane) when an organic P substrate (para-nitrophenyl phosphate) is present, though it is difficult to remove all soil particles completely. The assay is similar to the soil phosphatase assay, but root phosphatase assays differ in the buffer, substrate concentration, and terminator solution used. Briefly, we took 0.5 - 1 g of fine roots < 1 mm and washed roots in milliQ water multiple times to remove as much soil as possible prior to adding roots to 9 mL of 50 mM sodium acetate-acetic acid (for 1 L: 2.88 g sodium acetate; pH = 5.0 using acetic acid). We added 1 mL of the substrate, 50 mM pNPP (1.856 g pNPP in 100 mL sodium acetate buffer), prior to incubation. The solution was terminated by removing 0.5 mL and adding it to 4.5 mL of 0.11 M NaOH. Absorbance was read at 405 nm. We made the standard curve for both assays using 0, 100, 200, 400, 1000 µM pNP.

Statistical analysis

Our data was considered on both a soil mass and soil volume basis using bulk density. Fine-root mass density was calculated by dividing the mass of roots in each depth increment by the volume of the depth increment. We then used fine root density to convert root phosphatase measurements originally expressed on a per g root basis to per soil volume.

We analyzed all data using R version 3.6.1 (R Development Core Team, 2011) and tested for normality with the Shapiro-Wilks test and for homogeneity of variances using the Levene's test. Non-normal data was rank transformed prior to performing a two-way ANOVA with Site and Soil Depth as factors and using the R package 'car'. For comparing soil and root PME, we used a two-way repeated measures ANOVA with an $\alpha = 0.05$ to denote statistical significance of

the factors and their interactions. We applied Tukey's HSD to analyze main effects and interactions using the R package 'lsmeans'.

We used the Spearman correlation coefficient to test correlation among variables. Stepwise multiple linear regression of soil and root PME used all variables as potential predictors except soil depth because ideally all other predictors could determine PME activity throughout the soil profile. We tested interactions among predictors, eliminated nonsignificant relationships ($\alpha = 0.05$), and used second-order Akaike information criterion (AICc) to determine the final models for both soil and root PME. Lastly, we assessed the importance of final predictors using standardized regression coefficients.

All data are publicly available from the NGEE-Tropics data archive (Norby et al., 2019) and summary data are presented in the Supplement.

Results

Soil conditions

Gravimetric soil moisture decreased, while bulk density increased with soil depth (**Figure 3.1 A, B; all tables and figures are in the Appendix**). Both soil moisture and bulk density were similar across sites within the top 20 cm except at ICV, which had the lowest soil moisture in surface soils (**dataset,** Norby 2019). Topography influenced differences in soil moisture and bulk density below 20 cm in the soil profile. Ridge sites (EVR and ICR) had lower soil moisture content and higher bulk density relative to valley sites (EVV and ICV), which contained higher soil moisture content and lower bulk density. Soil moisture was strongly negatively correlated with bulk density **(**$r = -0.94$; $p < 0.05$; **Figure 3.2**).

Clay and sand fraction patterns were opposite between El Verde and Icacos sites (**Figure 3.1 C, E**). Clay fraction in El Verde (33-59%) was much higher than in Icacos (12-31%) (**Figure 3.3**). In contrast, Icacos soils were dominated by sand, 34-56% versus 7-25% in El Verde. All sites contained roughly similar amounts of silt (~ 40%; **Figure 3.1 D**) and neither silt nor sand varied with depth (**Figure 3.1 C, E**). Although, clay fraction (0-5 cm) at the surface did significantly differ from deeper soil layers; site was still the primary driver of variation in clay fraction.

Soil phosphorus

Total soil P, organic P, and resin P were highest in El Verde relative to Icacos with no

observable differences due to topography (**Figure 3.1 F, G, H)**. Generally, concentrations of organic P and total soil P were two times higher in El Verde, though differences in resin P (available P) were more pronounced. ICR had the lowest concentrations of resin P (0.07 ± 0.02 g P g_{soil}^{-1}) less than half the concentration in ICV and 25% lower than the concentration in EVR (0.43 ± 0.06 g P g_{soil}^{-1}; **(Table 3.1**). Concentrations of total, organic, and resin P differed between ICR and ICV, but not between EVR and EVV. Total soil P and resin P did not vary with depth, but concentrations of organic P were highest in surface and deep layers and lower at intermediate depths.

Fine roots throughout the soil profile

Fine-root (< 1 mm in diameter) mass density decreased with depth (**Figure 3.4 A**). On average, 82% of fine root mass density at all sites was in the top 15 cm. Average fine root density from all sites declined by over 80% from surface to deep soils (dataset; Norby 2019). Similarly, phosphorus concentration in fine roots decreased with depth (**Figure 3.5**). However, specific root surface area (SRA) and specific root length (SRL) of fine roots, which we measured only within the top 30 cm of the soil profile, did not decrease with depth or differ among sites (**Figure 3.4 B)**.

Soil phosphomonoesterase

Soil PME declined precipitously with depth, except at ICV, and was influenced by site (**Figure 3.6 A**). Soil PME at 80-90 cm averaged across all sites (2.61 ± 0.92 µmol pNP g_{soil}^{-1}) was approximately 85% lower than soil PME activity at 0-5 cm (17.42 ± 2.15 µmol pNP g_{soil}^{-1}) (dataset; Norby 2019). There were no differences in soil PME activity when the same depth was compared between two different sites, suggesting that plant and soil factors that varied with depth were strong drivers of soil PME throughout the soil profile.

Stepwise multiple linear regression using all data from the entire soil profile indicated that fine root mass density and total soil P explained 57% of the variation in soil PME ($p < 0.05$; **Figure 3.6 B**). Using standardized regression coefficients, fine root mass density emerged as the primary driver influencing soil PME, accounting for approximately 35% of variation (**Table 3.2).**

Root phosphomonoesterase

Root PME did not decline in 30 cm of the soil profile (**Figure 3.7 A**) and was on average 9% lower in El Verde than in Icacos (**Table 3.3**). Within Icacos, root PME activity was highest

in ICR (98.86 ± 14.57 mol pNP g_{root}^{-1}) than at ICV. Specific root length (SRL) and resin P concentration explained 79% of root PME variation in a multiple linear regression ($p < 0.05$; **Figure 3.7 B)**. The standardized regression coefficient for SRL was higher than for resin P, indicating that a higher proportion of the variation in root PME was explained by SRL (~ 68%) as compared to resin P (~11%) (**Table 3.4**).

Root and soil phosphomonoesterase per soil volume

Soil PME expressed per soil volume was consistently two orders of magnitude higher than root PME expressed per soil volume across all sites and depths (**Figure 3.8**). Soil PME was influenced most by site ($F_{Site} = 10.767$; $p < 0.05$), whereas root PME was influenced by both site and depth ($F_{Site} = 4.007$; $F_{Depth} = 5.15$; $p < 0.05$) (**Table 3.5**). In two-way repeated measures ANOVA, soil PME was significantly higher than root PME at all depths, with site modifying those differences in activity (**Table 3.5 C**). There was no clear linear correlation between root and soil PME activity. However, the relationship between them appears to be nonlinear. Furthermore, root PME was strongly correlated with SRL unlike soil PME (**Figure 3.9**).

Discussion

A major uncertainty in understanding and modeling the response of tropical forests to altered climates is capturing the feedbacks between root traits and soil factors that influence the P cycle and ultimately, plant productivity. Mineralization of organic P phosphomonoesters is mediated by phosphomonoesterase (PME) enzymes produced by roots (root PME) and microbes (soil PME), constituting an important role in increasing P availability in tropical forests occurring on P-limited soils (Nannipieri et al., 2011; Reed et al., 2011; Lambers et al., 2015; Dalling et al., 2016). However, root and soil factors that mediate the expression of PME are not well understood, particularly as these factors change throughout the soil profile.

We found that soil PME does decrease with depth, but not root PME unless root PME is expressed per soil volume, accounting for changes in fine-root mass density. As we expected, there was a difference in which root traits and soil factors influenced root or soil PME. We correctly predicted that root PME would be sensitive to resin P and specific root length, but we found that soil PME was not responsive to organic P alone, but rather both total P and fine-root mass density. Our results indicate that a combination of root and soil factors were necessary to accurately predict both root and soil PME. Our data can constrain estimates of biochemical mineralization throughout depth as affected by existing root distribution structures and soil P

variables.

Fine-root mass density strongly influenced soil and root PME throughout depth

Soil PME down to 1 m decreased with depth (Fig. 3A), mirroring the decline in fine root mass density (**Figure 3.4 A**). Root biomass in P-limited areas tend to be concentrated in the upper soil layers where available P concentrations are generally higher (Lynch & Brown, 2001). Furthermore, the rhizosphere is often considered a 'hotspot' of biological activity owing to root exudation of carbon compounds that attract and sustain an active microbial community. Thus, as fine roots and associated microbial activity decline; soil PME declines as well. Prior studies also confirm that soil PME tends to be higher in surface layers where biological activity and fine roots are much more prevalent (Harrison, 1987; Nannipieri et al., 2011; Margalef et al., 2017). Our average soil PME across all sites and depths was slightly higher at 9.4 μmol pNP $gsoil^{-1}$ hr^{-1} than the global average of 8.8 μmol pNP $gsoil^{-1}$ hr^{-1} in a global survey of tropical and subtropical forests (Margalef et al., 2017).

Unlike soil PME, root PME only declined with depth when it was expressed per soil volume. Root PME on a soil volume basis also decreased due to a decline in fine root mass density at 20-30 cm compared to 0-10 cm (**Figure 3.4 A**). Furthermore, expressing soil and root PME per soil volume resulted in higher levels of soil PME than root PME. However, this is not surprising as root PME has low mobility in soils and is mostly restricted to the rhizosphere which occupies a smaller proportion of the soil volume (Yadav & Tarafdar, 2001).

Soil phosphomonoesterase was predicted by fine-root mass density and total P

Fine-root mass density and total soil Pw ere the two most important factors in predicting soil PME activity. Fine root mass density likely regulates soil PME because roots are a source of PME and because higher amounts of fine roots relate to increased microbial activity. Tarafdar and Jungk (1987) measured soil PME in 1 mm rhizosphere soil increments and found a concurrent decline in soil PME and fungal and bacterial populations with increasing distance from the root surface (Helal, 1990; Finzi et al., 2015). However, our result that total P, not organic P, was predictive of soil PME contradicts reports that find stronger correlations between organic P and soil PME (Margalef et al., 2017; Turner & Haygarth, 2005). The response to total soil P, rather than to organic P may have been due to the activity of soil PME enzymes that remained stable in the soil, insensitive to fluctuations in substrate availability and root or microbial demand though there was a correlation between total P and organic P (Burns et al.,

2013; Nannipieri et al., 2011).

Root phosphomonoesterase was predicted by specific root length and resin P

Physical characteristics of roots, like specific root length and the proliferation of roots throughout the soil profile, influence the spatial distribution of root PME activity in the soil volume. Fluorescent images of root PME establish that root PME is largely restricted to the root surface (Spohn and Kuzyakov, 2013), confirming earlier research based on 32P (Tarafdar & Jungk, 1987). Generally, thinner and longer roots with correspondingly high surface areas are associated with greater soil phosphatase activity (Kong et al., 2014, 2017). Similarly, we found that SRL corresponded to higher levels of root PME, consistent with current research (Kitayama, 2013; Lugli et al., 2019; Ushio et. al, 2010).

Previous work at these same sites showed that root PME strongly decreased with resin P (Cabugao et al., 2017). Here, the negative correlation between root PME and resin P was not significant. However, the emergence of resin P as a strong predictor of root PME still links root PME to fluctuations in available P (Helal & Dressler, 1989), as found in other studies where phosphatase is suppressed by P fertilization (Olander & Vitousek, 2000; Yadav & Tarafdar, 2001; Allison & Vitousek, 2004; Zheng et al., 2015). Our results show that soil P measurements and root data were strong predictors of both soil and root PME, emphasizing the need to understand root traits and root function within the context of soil P to better define and model the influence of roots and phosphatase enzymes on organic P mineralization.

Soil and root characterization are both necessary to improve P representation in ecosystem models

Biochemical mineralization in models that include the P cycle is generally a fixed parameter independent of observed relationships between roots, microbes, and organic P (Achat et al., 2016). When roots are included, root distribution and nutrient uptake also occur as fixed parameters or as a proportion of C allocated from photosynthesis (Koven et al., 2013; Smithwick et al., 2014; Warren et al., 2015). These approaches exclude the involved role of root systems in actively shaping the P cycle, particularly through the production of phosphatase enzymes which increase P supply for plant uptake. Our results can help constrain model estimates of biochemical mineralization by providing root and soil PME measurements with concurrent changes in soil P and root distribution throughout depth. Furthermore, our finding that root and soil PME rely on both soil P data and fine-root traits strengthens the need to represent the amount of absorptive

roots as opposed to treating all root mass as functionally similar given that root mass does not necessarily reflect total absorptive area of the root system (Hodge 2004).

Conclusion

Phosphatase released by both microbes and roots is a mechanism to recycle P locked in organic compounds to available P needed to maintain productivity in tropical forests occurring on P-limited soils. We found that both soil P and root trait data were necessary to predicting root and soil PME. Soil PME was positively correlated and best predicted by total soil P and fine-root mass density. The most important factors for root PME were resin P, which was negatively correlated with root PME, and specific root length, which was positively correlated with root PME. Lastly, the decline of root and soil PME down the soil profile mirrored the decline in fine-root mass density, highlighting the important role of the root system in determining enzyme activity. Altogether, our findings illustrate that organic P mineralization by phosphatase enzymes down the depth profile depends on understanding fine-root traits within the context of soil P. For a plant, P availability is not solely a function of the concentration of available P in the soil, but also the capacity of roots to change in order to find and increase available P. Ultimately, root traits and root functions, like phosphatase, are critical facets of belowground biogeochemistry that in part regulate feedbacks between P availability and tropical forest productivity.

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Appendix

Table 3.1 Total soil phosphorus (A), organic phosphorus (B), and resin P concentrations (C) averaged across all depths at each site for the 1 m core. SEM refers to standard error of the mean while SD is the standard deviation.

A. Summary of total soil phosphorus content

Site	Total soil P mean (mg/g)	Total soil P SEM	Total soil P SD
EVR	0.433	0.061	0.250
EVV	0.398	0.036	0.150
ICR	0.113	0.019	0.080
ICV	0.176	0.033	0.141

B. Summary of organic phosphorus content

Site	Organic P mean (mg/g)	Organic P SEM	Organic P SD
EVR	0.231	0.021	0.085
EVV	0.262	0.021	0.091
ICR	0.075	0.007	0.031
ICV	0.116	0.011	0.045

C. Summary of resin phosphorus content

Site	Resin P mean (μg/g)	Resin P SEM	Resin P SD
EVR	0.429	0.064	0.264
EVV	0.411	0.046	0.184
ICR	0.074	0.017	0.074
ICV	0.201	0.066	0.272

Table 3.2 Soil phosphomonoesterase multiple linear regression summary table (A) and summary table with standardized regression coefficients (B).

A. Multiple linear regression table

	Coefficient	Std. Error	t-value	p-value
Intercept	-0.155	2.286	-0.068	0.947
Total Soil P	18.576	6.162	3.015	< 0.05
Fine root mass density	9.589	2.280	4.206	< 0.05

Residual standard error: 4.717 on 20 degrees of freedom

B. Multiple linear regression standardized regression coefficients

	Coefficient	Std. Error	t-value	p-value
Intercept	-2.084×10^{-16}	1.434×10^{-1}	0.000	1.000
Standardized Total Soil P	4.420×10^{-1}	1.466×10^{-1}	3.015	< 0.05
Standardized fine root mass density	6.167×10^{-1}	1.466×10^{-1}	4.206	< 0.05

Residual standard error: 0.6876 on 20 degrees of freedom

Table 3.3 Root phosphomonoesterase (PME) summary table by site (n =3). SEM refers to standard error of the mean while SD refers to standard deviation.

Site	**Root PME mean** (μmol pNP g_{root}^{-1})	Root PME SEM	Root PME SD
EVR	59.08	7.76	23.27
EVV	53.67	0.39	26.55
ICR	98.86	14.57	43.71
ICV	25.41	4.01	12.02

Table 3.4 Root phosphomonoesterase (PME) multiple linear regression summary table (A) and summary table with standardized regression coefficients (B).

A. Summary of multiple linear regression for root phosphomonoesterase

	Coefficient	Std. Error	t-value	p-value
Intercept	-0.673	25.961	-0.042	0.967
Resin P	-76.600	24.838	-3.084	< 0.05
Specific root length	5.092	0.886	5.747	< 0.05

Residual standard error: 13.25 on 9 degrees of freedom

B. Summary of root phosphomonoesterase multiple linear regression standardized regression coefficients

	Coefficient	Std. Error	t-value	p-value
Intercept	-2.084×10^{-16}	1.434×10^{-1}	0.000	1.000
Standardized Total Soil P	4.420×10^{-1}	1.466×10^{-1}	3.015	< 0.05
Standardized fine root mass density	6.167×10^{-1}	1.466×10^{-1}	4.206	< 0.05

Residual standard error: 0.6876 on 20 degrees of freedom

Table 3.5 ANOVA tables for soil phosphomonoesterase per soil volume (A) and root phosphomonoesterase (B) and results of a two-way repeated measures ANOVA to test differences between both enzymes (C).

A. Soil phosphomonoesterase per soil volume ANOVA

	Sum of Squares	Degrees of Freedom	F-value	p-value
Intercept	2408.33	1	100.8140	< **0.05***
Site	771.67	3	10.767	< **0.05***
Depth	11.56	2	0.241	0.787
Site:Depth	188.26	6	1.313	0.289
Residuals	573.33	24		

B. Root phosphomonoesterase per soil volume ANOVA

	Sum of Squares	Degrees of Freedom	F-value	p-value
Intercept	2133.33	1	43.200	< **0.05***
Site	593.67	3	4.007	< **0.05***
Depth	509.56	2	5.159	< **0.05***
Site:Depth	486.72	6	1.642	0.179
Residuals	1185.17	24		

C. Two-way repeated measures ANOVA

	Sum of Squares	Mean Squares	Degrees of Freedom	F-value	p-value
Site	2213.8	737.9	3	9.69	< **0.05***
Enzyme Type	19672.6	19672.6	1	258.491	< **0.05**
Site:EnzymeType	1016.4	338.8	3	4.45	< **0.05***
Depth	277.5	138.7	2	1.82	0.196

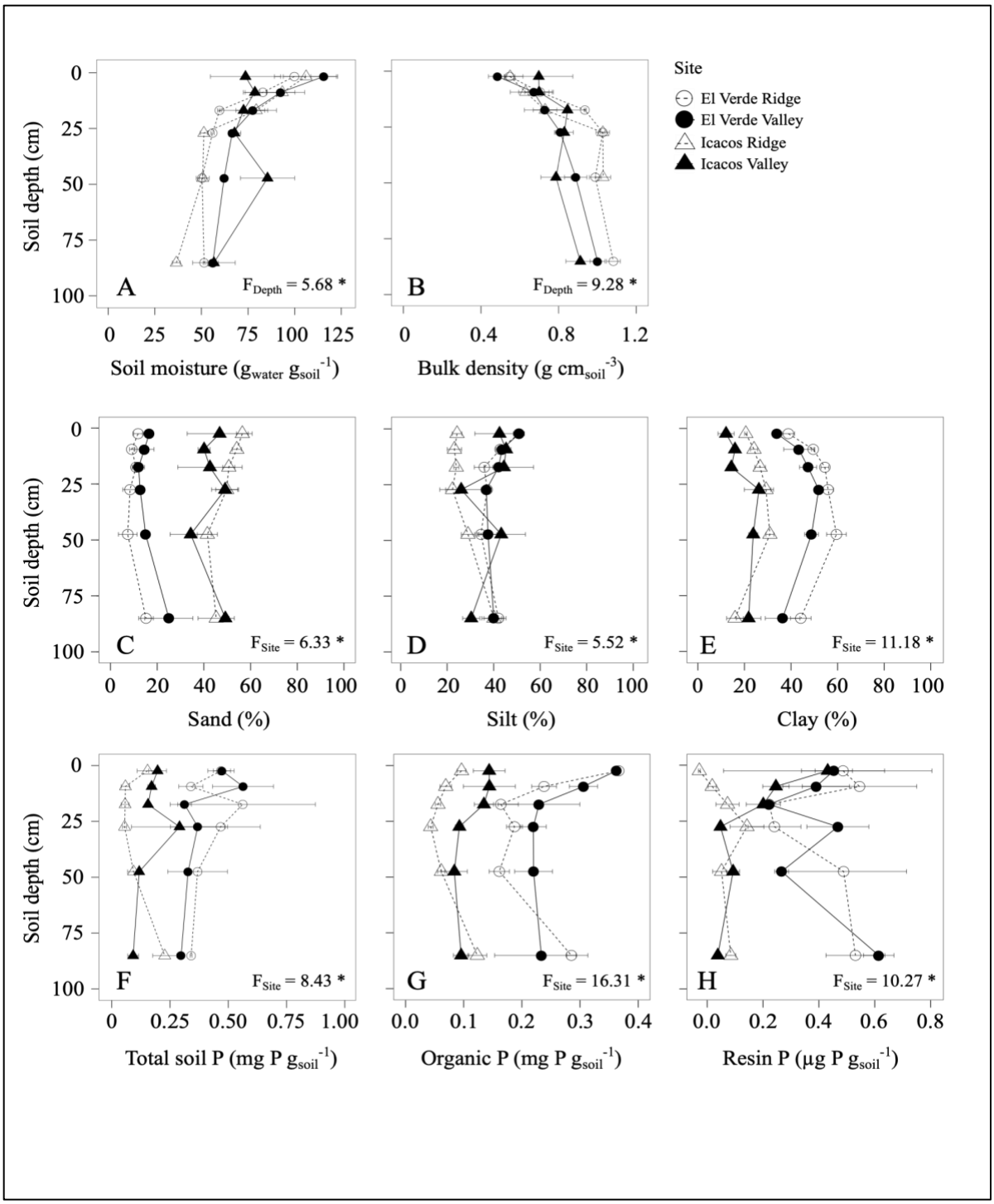

Figure 3.1 Average of all three cores (n=3) taken at each site and depth for: soil moisture (A), bulk density (B), sand fraction (C), silt fraction (D), clay fraction (E), total soil P (F), organic P (G), and resin P (H). Error bars are standard error of the mean and F-values represent significant differences due to either site or depth of ranked transformed variables in a two-way ANOVA.

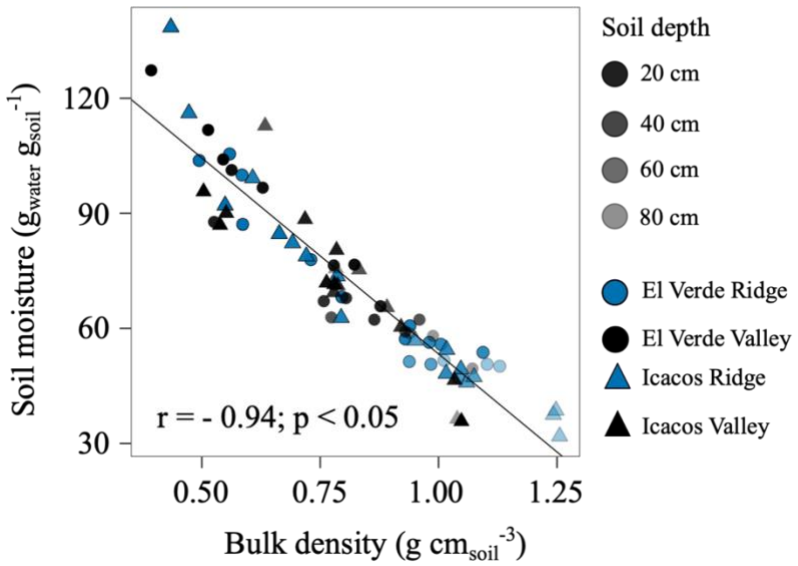

Figure 3.2 Soil moisture and bulk density correlation across all sites and depths.

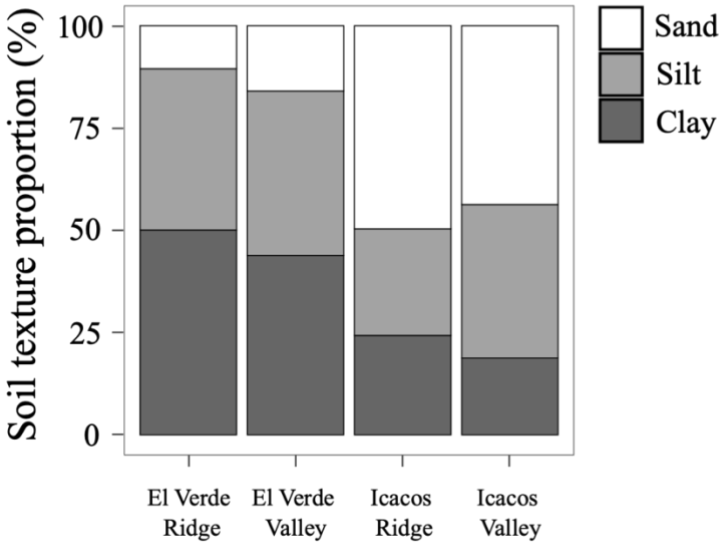

Figure 3.3 Sand, silt, clay fractions by site (n = 3). Averages were taken across all depths.

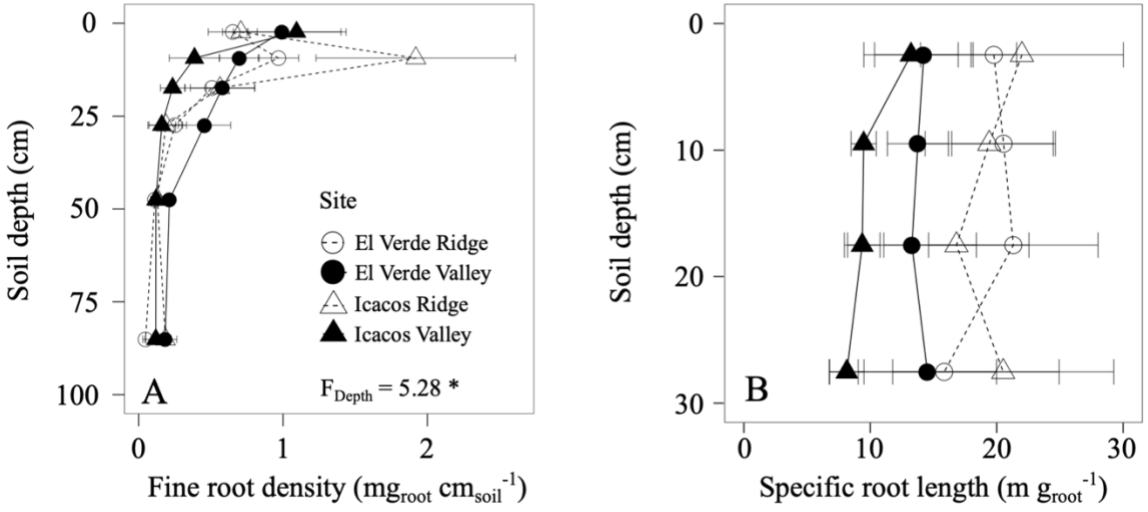

Figure 3.4 Average of all three cores (n=3) taken at each site and depth for: fine root mass density (A) and specific root length (B). Error bars are standard error of the mean and F-values represent significant differences due to either site or depth of ranked transformed variables in a two-way ANOVA. Specific root length was only measured from 0 - 30 cm.

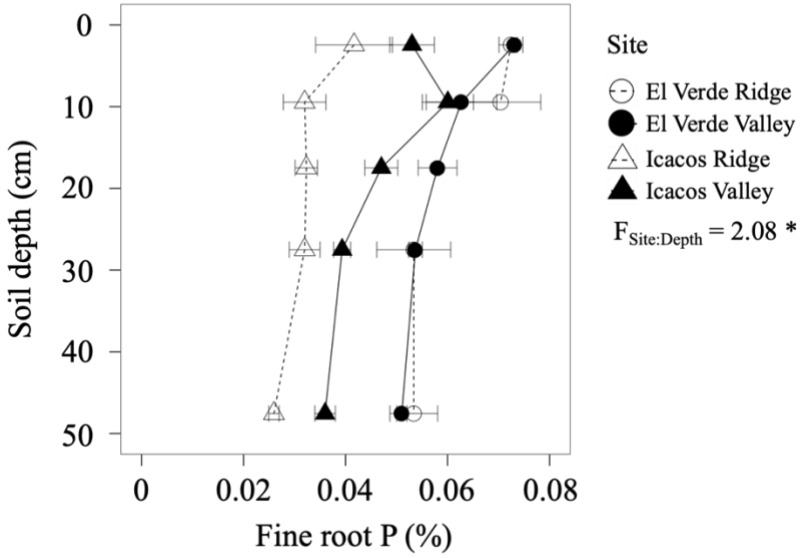

Figure 3.5 Percent of phosphorus per g dry root. Error bars represent standard error of the mean (SEM) and full table of values are in **Table 3.1**.

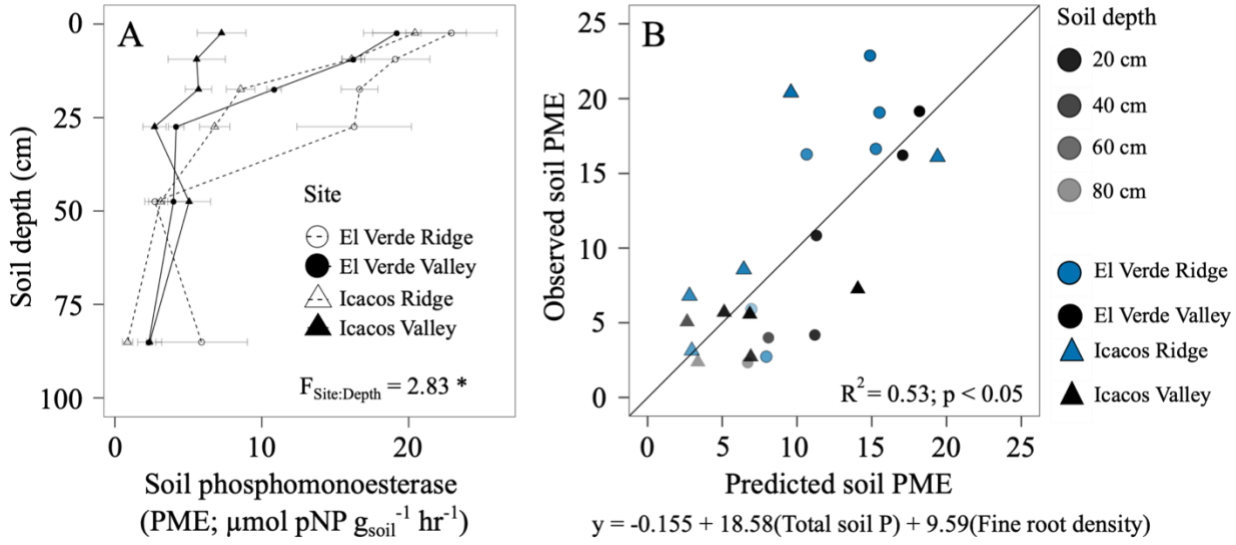

Figure 3.6 Soil phosphomonoesterase (PME) with points representing the mean of three locations sampled at each site (n = 3) and error bars representing standard error or the mean (A). F-values represent significant differences due to either site or depth of ranked transformed variables in a two-way ANOVA. Correlation between predicted soil PME from stepwise multiple linear regression and our observed values of soil PME **(B)**.

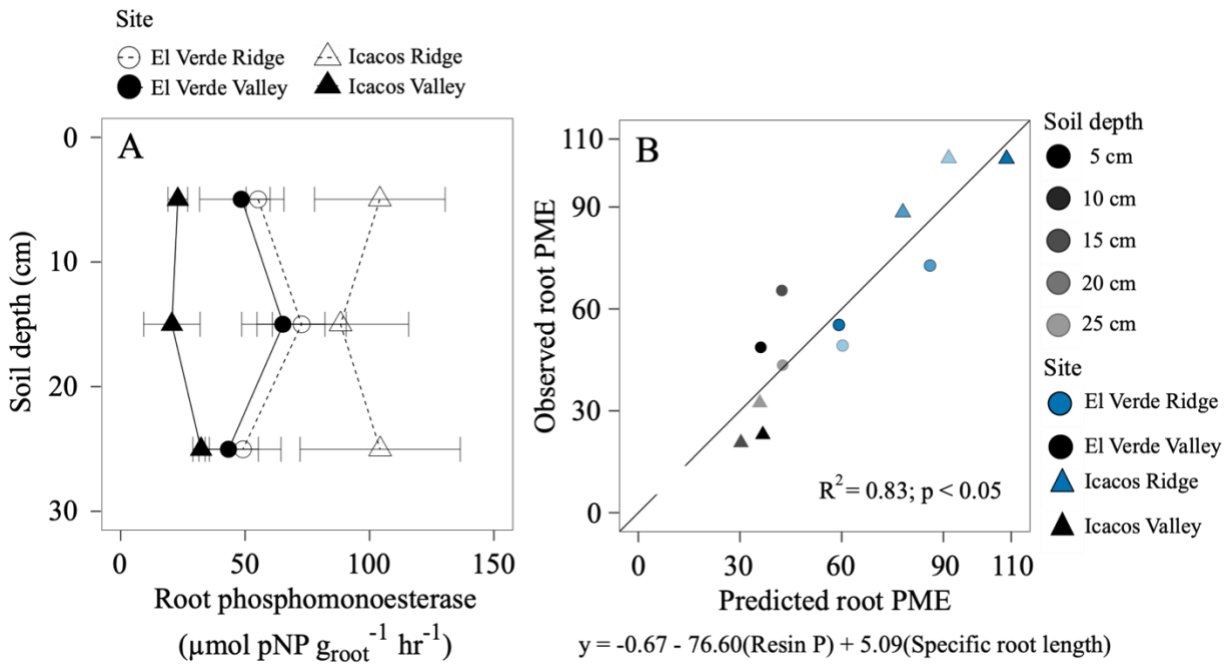

Figure 3.7 Average root PME within the upper 30 cm of the soil profile of three soil cores taken at each site (n =3). Error bars represent standard error of the mean (A). Predicted specific root PME vs. observed specific root PME from stepwise multiple linear regression **(B)**.

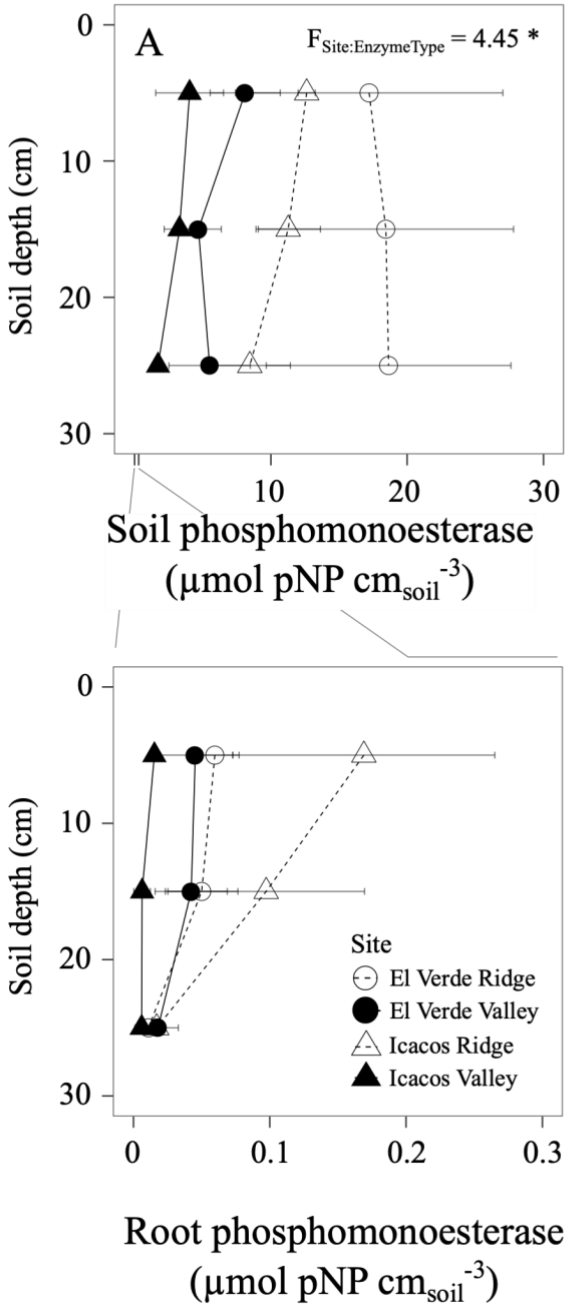

Figure 3.8 Root (A) and soil (B) PME expressed on soil volume basis. Bars represent averages of three cores taken at each site (n =3) with error bars as standard error of the mean. F-values represent significant differences based on ranked transformed variables in a linear mixed model.

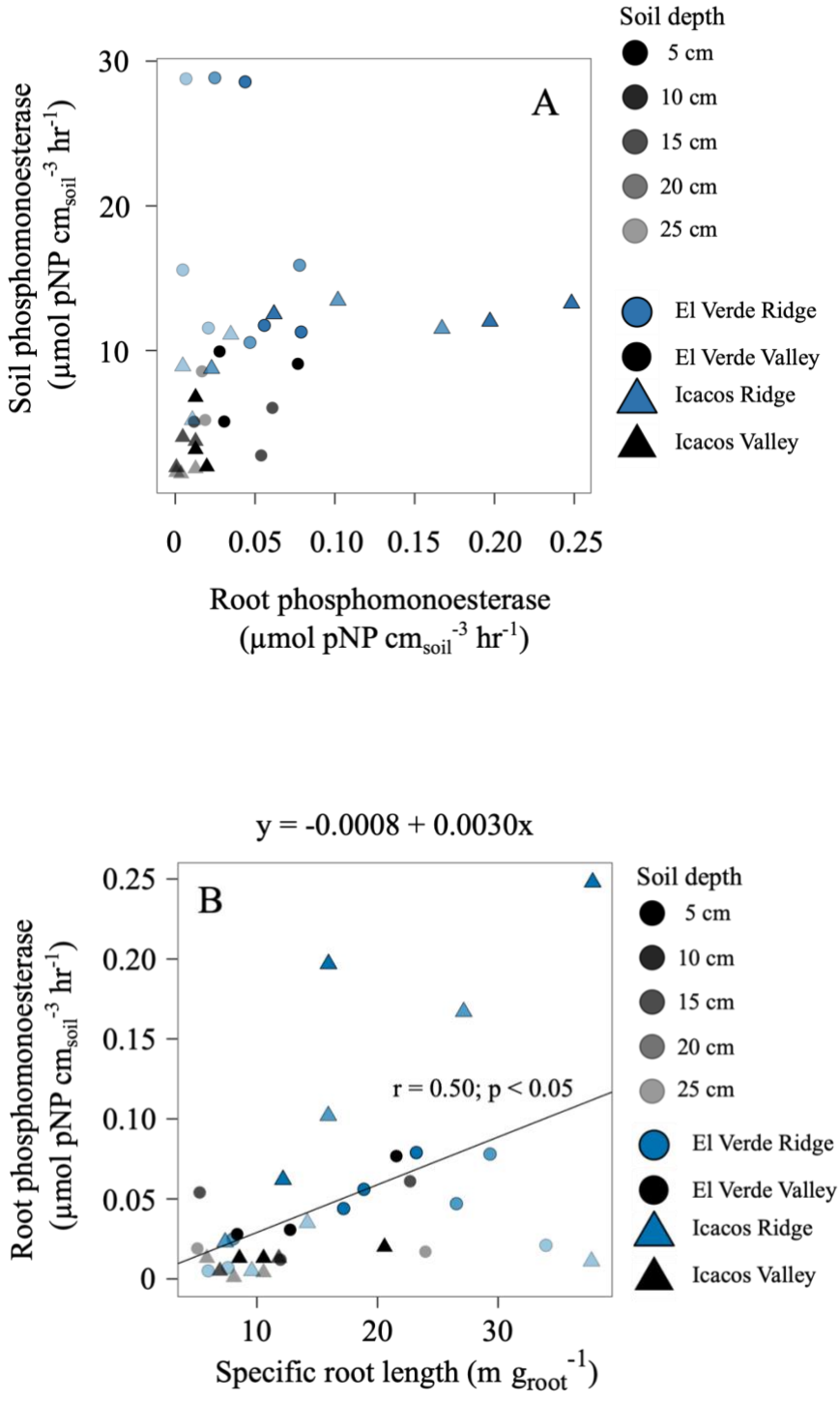

Figure 3.9 Correlations between soil and root phosphomonoesterase expressed per soil volume (A), soil phosphomonoesterase and specific root length (SRL) (B), and root phosphomonoesterase and SRL (C).

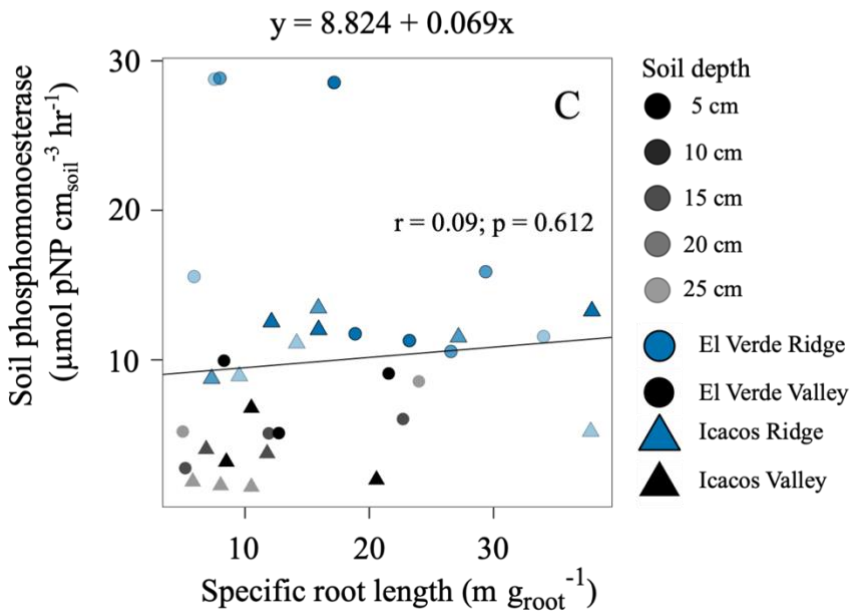

Figure 3.9 continued Correlations between soil and root phosphomonoesterase expressed per soil volume (A), soil phosphomonoesterase and specific root length (SRL) (B), and root phosphomonoesterase and SRL (C).

Chapter 4

Root phosphomonoesterase increases in tropical seedlings grown under elevated [CO_2] in phosphorus-limited soils

My use of "we" in this chapter refers to my co-authors and myself. This chapter is an iteration of a manuscript currently being prepared by co-authors Kristine Grace M. Cabugao, Martijn Slot, Klaus Winter, Kristin Saltonstall, Benjamin L. Turner, Jorge Aranda, Aurelio Virgo, David J. Weston, Richard J. Norby to be *submitted* to *Plant and Soil* in June 2020.

Abstract

The tropical carbon sink holds a quarter of the world's terrestrial carbon stock and exchanges the largest amount of carbon with the atmosphere among the terrestrial ecosystems. A critical uncertainty in understanding and predicting climate change is whether the tropics will remain carbon sinks through the enhancement of forest growth with rising atmospheric CO_2. However, phosphorus has the potential to limit growth responses because phosphorus is a vital component of energy metabolism, nucleic acid synthesis, and phospholipid membranes though often occurs in limiting concentrations in tropical soils. Thus, plants must rely on the mineralization of P from organic P, which comprises a much larger portion of soil P, to meet demand. P mineralization by phosphatase enzymes released by roots and microbes, will likely play a key role in regulating the magnitude of P limitation constraints under eCO_2. However, it is not clear whether phosphatase responds to eCO_2, especially in different tropical tree species on P-limited soils. To test how phosphatase in roots and rhizosphere soil responded to elevated CO_2, we exposed seedlings of four different tree species: *Inga spectablis, Adenanthera pavonina, Tabebuia guayacan, Tabebuia rosea* to either 400 ppm CO_2 (Ambient treatment) or 800 ppm CO_2 (Elevated treatment). After three months of growth, we harvested roots and rhizosphere soil for phosphatase measurements and preserved a portion of rhizosphere soil for microbial community analysis. Consistent with other elevated CO_2 studies, we found that belowground biomass increased between Ambient and Elevated treatments, though large differences between Ambient and Elevated plants only occurred in *T. guayacan*. Similarly, root phosphomonoesterase, but not soil phosphatase, significantly increased under eCO_2, though only in *T. guayacan* and *A. pavonina*. We suspect that variation in root phosphatase activity may be due to how different seedlings of the varying tree species utilized available P within the soil

Introduction

Tropical forests drive global fluxes of carbon, simultaneously constituting a significant carbon sink owing to unparalleled levels of productivity among terrestrial forests (Pan et al., 2011). A critical uncertainty in predicting climate change is whether elevated [CO_2] will enhance productivity of tropical forests, enhancing the size of the tropical carbon sink. Experimental evidence consistently demonstrates that elevated [CO_2] increases photosynthesis, growth, and aboveground biomass in both temperate and tropical ecosystems (CO_2 fertilization effect) (Ainsworth & Long, 2005). However, a significant caveat is the assumption of optimal nutrient availability and by extension, an effective root system capable of acquiring those nutrients necessary to supporting plant growth and productivity.

Nutrient limitation could constrain growth in elevated [CO_2] (Jin et al., 2015). Experiments in temperate ecosystems demonstrated an initial increase in growth until the onset of N limitation (Norby et al., 2016; Norby & Zak, 2011). The observation that nutrient limitation, particularly of N, can and does limit growth despite abundant supplies of CO_2 is well established experimentally (Chambers & Silver, 2007; Luo et al., 2004). However, the impact of P limitation, which is generally considered the limiting nutrient in tropical forests and how it may contribute to moderating plant growth under elevated [CO_2] is still not well understood.

Orthophosphate, the inorganic form of phosphorus (P) necessary to plant growth is essential for plant energy metabolism, nucleic acid synthesis, and cell wall membranes (Jin et al., 2015). However, tropical soils are characteristically low in orthophosphate. Prolonged weathering, strong sorption, and rapid immobilization of orthophosphate by microbes are among the physical, chemical, and biological processes that inhibit P availability (Achat et al., 2013; Sayer & Banin, 2016; Walker & Syers, 1976). Thus, sustaining growth and productivity of tropical forests under elevated [CO_2] may depend on how trees manage P availability (Jin et al., 2015).

The annual profusion of growth in tropical forests despite minimal availability of orthophosphate relative to temperate forests suggest that tropical trees either use P efficiently or acquire P effectively (Dalling et al., 2016; Vitousek, 1984). Leaf data indicated that P resorption was much higher in tropical versus temperate trees and that on average, tropical leaves contained less P despite similar if not greater photosynthetic C assimilation than their temperate counterparts (Vitousek, 1984). Although tropical trees may use P more efficiently, increasing

growth to keep pace with increasing CO_2 will require more P to create new tissues and C sinks. Thus, to support growth, trees must acquire more P, which relies entirely on the root system.

Phosphorus acquisition is inherently determined by the root system, as fine roots are the main interface between trees and soil ecosystems (Gregory, 2006). Elevated [CO_2] reliably increases plant C allocation to belowground processes such as respiration, root growth, and exudation owing to the increased demand for water and nutrients (Finzi et al., 2007; Lynch & St.Clair, 2004). Indeed, elevated [CO_2] stimulates root growth more than other plant organ (Norby et al., 1999; Iversen et al., 2008; Norby et al., 2004, 2010; López-Arredondo et al., 2014; Jin et al., 2015). Plants have many mechanisms to access P in the soil such as root proliferation in nutrient patches, mycorrhizal associations that increase absorbing surfaces, and modification of the rhizosphere (Lambers, 2006; Richardson & Simpson, 2011; Jin et al., 2015). Modifications to the rhizosphere include the release of protons to acidify the rhizosphere, encouraging desorption of orthophosphate (Ahemad & Kibret, 2014). Additionally, carboxylate exudation minimizes the fixation of orthophosphate into iron and aluminum complexes through ligand exchange and can also maintain P concentration via chelation (Ahemad & Kibret, 2014). While these methods shift chemical and sorption conditions to target orthophosphate already in the soil, plants can also release phosphatase enzymes that mineralize organic P into orthophosphate. Understanding how elevated [CO_2] affects the activity of phosphatase exuded from roots remains a key challenge to determining the flexibility of a key facet of P acquisition that may in part regulate how tropical forests respond to elevated [CO_2] (Reed, Yang, & Thornton, 2015; Fleischer et al., 2019; Yang et al., 2019).

Enzymes that catalyze the removal of orthophosphate from organic P compounds –phosphatases– are crucial to supplying plants and microbes with P necessary to grow and develop (Dalling et al., 2016). Of the types of phosphatase enzymes, each distinguished by their substrate, phosphomonoesterase (PME; E.C. 3.1.2) is the most widely studied in soils (Nannipieri et al., 2011). However, it should be noted that PME is one among many and is considered to act sequentially after phosphodiesterase (PDE), which breaks down phosphodiester compounds into an orthophosphate ion and a phosphomonoester compound (Turner & Haygarth, 2005). Phosphomonoesterase frees the orthophosphate group in phosphomonoester compounds such as mononucleotides and sugar phosphates, which can constitute a significant proportion of soil organic P compounds (Nannipieri et al., 2011; Turner & Engelbrecht, 2011). Root functions,

such as the exudation of compounds and enzymes that influence soil conditions are an important facet of P acquisition that could influence plant acclimation to changing conditions. However, PME activity and root function in general are not often considered. Thus, whether plants and microbes can adjust phosphatase activity to support growth under elevated [CO_2] is a huge uncertainty in predicting and modeling future tropical forest growth.

Model simulations of the tropical C sink indicate that excluding P overestimates its size (Reed et al., 2015; Wang, Houlton, & Field, 2007), and that P mineralization, especially the role of phosphatase enzymes, will play a key role in regulating the magnitude of P limitation constraints over elevated [CO_2] (Fleischer et al., 2019; Yang et al., 2014; Yang et al., 2016). To test the influence of CO_2 on phosphatase activity and growth, we grew seedlings of four tree species (*Inga spectablis*, *Adenanthera pavonina*, *Tabebuia rosea*, *Tabebuia guayacan*) in glasshouses with a CO_2 concentration of either 400 ppm for the controls or 800 ppm to simulate elevated CO_2 over three months. Our main hypothesis was that root PME activity would increase in the elevated [CO_2] chamber relative to the control, but not soil PME activity, which may not be as influenced by plant growth.

Materials and Methods

Study species and growth conditions

Seeds from *Adenanthera pavonina*, *Tabebuia rosea*, *Tabebuia guayacan*, and *Inga spectablis* were collected on April 2017. We chose to collect two tree species that associated with N_2-fixing microbes: *A. pavonina* and *I. spectablis* and two tree species that did not: *T. rosea* and *T. guayacan*. However, *A. pavonina* and *I. spectablis* did not have nodules when harvested, though they have in previous experiments in similar conditions (Nasto et al., 2019).

We planted four to five seeds within 12.7 cm by 30.5 cm pots with a 5 L volume (CP512; Stuewe & Sons, Inc.). The pots were filled with an 80:20 soil to sand. Soil used came from a site in Santa Rita classified as an Ultisol soil occurring on pre-Tertiary basalt. Average resin-extractable P is 0.13 mg P kg^{-1} with a total exchangeable base concentration of 1.4 cmol kg^{-1}. We germinated seeds in open-air shaded tables without fertilizer for three weeks prior to moving all the seedlings into glass chambers. Additionally, 12 pots containing just the soil: sand mixture were used as a control for each chamber. Every morning and evening, we watered seeds with approximately 50 mls distilled water. The glasshouse temperature were maintained by split air-condition units that matched outdoor temperatures (approximately 32 ℃ daytime and 24 ℃)

while CO_2 concentrations were maintained at approximately 400 ppm for the Ambient CO_2 chamber and at approximately 800 ppm for the elevated CO_2 chamber using a GMW21D carbon dioxide transmitter (Vaisala) and a CR-5000 measurement and control system (Campbell Scientific). During the experiment, we switched plants and treatments from one chamber to another twice to minimize chamber influence on plant growth.

Photosynthetic capacity

We measured net photosynthesis on a minimum of four individuals per tree species per CO_2 treatment. Photosynthetic activity was measured at an irradiance level of 1,000 µmol m^{-2} s^{-1} in both the ambient (400 ppm) and elevated [CO_2] (800 ppm) treatments. Saturating photosynthesis (Asat) was calculated from full A/Ci curves. For photosynthesis measurements, we used a LI-6400XT portable photosynthesis system (LI-COR) on fully expanded leaves. We used ImageJ to calculate leaf surface area from leaf photos for *A. pavonina*, *T. guayacan*, and *T. rosea* because fully expanded leaves did not fill except the 2 x 3 cm leaf cuvette. However, *I. spectablis* had leaves large enough to ensure that the area of the cuvette was filled.

Plant harvest and collection

We harvested plants after 3 months of growth with the exception of *I. spectablis*, which was harvested earlier due to its faster growth. We removed plants gently from their pots and excavated the root system. The first lateral root was shaken inside the rhizosphere soil collection bag prior to the roots being collected separately. Leaves and stems were placed in the same collection bag except for the leaf used for photosynthetic measurements, which was placed in its own bag. At the laboratory, leaves were excised from the stem at the base of each leaf, leaving the petiole attached to the stem. Leaves were then scanned for leaf area using an automated leaf area meter (LI-3000A; Li-COR) before being placed in envelopes to dry. Leaves and stems were dried separately for a minimum of three days. After being dried, leaves and stems were weighed separately. Field-moist root samples for root phosphomonoesterase (PME) assays were separated and the rest of the roots were dried and weighed. After the root PME assays, the roots were dried, weighed, and stored separately from the rest of the root samples to prevent contamination of non-assay roots with PME assay reagents. However, the dry weight of the assay roots was added to the dry weight of the non-assay roots.

Rhizosphere soil phosphatase activity

Soil phosphomonoesterase (PME) and phosphodiesterase (PDE) were assayed using 4-

methylumbelliferyl phosphate (MUP) and bis-(4-methylumbelliferyl) phosphate (bis-MUP) as a substrate respectively. We prepared the substrates by dissolving 12.81 mg of MUP or 20.72 mg of bis-MUP into 1 ml of methylcellosolve. The solutions were then diluted into 250 ml for a final concentration of 100 µM of substrate in the final assay solution. Methylumbelliferyl (MU) - the product of phosphatase hydrolysis of MUP or bis-MUP - standards were prepared by first dissolving 19.82 mg of methylumbelliferone in 100 ml of milliQ H_2O to create a stock standard. Then 0.5 ml of the stock standard was diluted with 50 ml of milliQ H_2O to have a final concentration of 10 nmol MU ml-1. An acetate buffer was prepared by dissolving 4.1 g sodium acetate in 200 ml of milliQ H_2O and adjusting the pH to 5.0 using concentrated acetic acid. The resulting solution was diluted to 250 ml with milliQ H_2O. Sodium azide for the blank wells and control plates was made by dissolving 0.65 g of sodium azide in 10 L of milliQ H_2O. Lastly, the reaction was terminated using a terminator solution made by dissolving 20g of NaOH in 500 ml of milliQ H2O before diluting it to 1 L.

Each 96-well black plate contained three rhizosphere soil samples, four columns for each sample. The four columns from left to right contained the blank, MU standard, MUP substrate, and bis-MUP substrate in a repeating pattern across the plate. All wells received acetate buffer, but blank wells received 100 µl of 1 mm sodium azide, MU standard wells received 100 µl of 10 nmol MU ml-1, MUP substrate wells received 100 µl of MUP, and bis-MUP substrate wells received 100 µl of bis-MUP. Control plates received the same solutions but did not contain any soil. A new control plate was made for each set of soil enzyme assays.

Approximately 2.00 ± 0.05 g of fresh soil was added to a 1 L plastic beaker with 200 ml of 1 mmol L-1 NaN3 and stirred for 5-mins. 50 µl of each soil suspension was added to their respective set of 4 columns before the plate was incubated at 26 ℃ along with the control which received only 50 µl of NaN3 in all wells. After 1 hr., 50 µl of 0.5 M NaOH was added to all wells in both soil plates and control plates in order to terminate the assay. Both soil plates and control plates were read on a fluorometric plate reader.

Root phosphomonoesterase activity

Root phosphomonoesterase activity was measured according to a modified version of Tabatabai and Bremner (1965). Briefly, the entire root system was washed three times with milliQ H_2O. Then 0.5 g of fine root (first three orders) were cut from various locations in the root system and placed in 9 ml of sodium-acetate buffer. After a 5-min equilibration time in a 27 ℃

water bath shaker, 1 ml of para-nitrophenyl phosphate (pNPP) was added before the entire solution was incubated for 1 hr. Control samples received 1 ml of buffer instead. Blanks were composed of buffer and 1 ml pNPP without roots. After 1 hr., enzyme activity of phosphomonoesterase was read on spectrophotometer and we calculated the intensity of the yellow color of para-nitrophenol (pNP), the product formed by the cleave of pNPP by PME released by the roots using a standard curve.

Statistical analysis

We used two-way ANOVA to test whether tree species, [CO_2] level, and the interaction between tree species and [CO_2] level affected root and soil phosphatase, biomass measurements, and photosynthesis. Data that were not normally distributed were ranked transformed prior to analysis. The strength of correlations between variables was assessed using Pearson's correlation tests. Statistical significance was determined using $\alpha = 0.05$. All data were analyzed in R (version 3.6.1).

Results

Elevated [CO_2] increased root biomass

Total biomass was not affected by elevated [CO_2] (**Figure 4.1 A**; **all tables and figures are in the Appendix**), though *I. spectablis* had the highest total biomass, followed by *A. pavonina*. Both *I.spectabilis* and *A. pavonina*, which are known to associate with N_2-fixers, had significantly higher biomass than *T. guayacan* and *T. rosea* ($F_{tree} = 39.5$; $p < 0.05$; **Table 4.1**), though no nodules were found at harvest. When total biomass was separated according to leaf, stem, or root mass; only root mass changed significantly between ambient and elevated [CO_2] treatments. Elevated [CO_2] increased root mass across all tree species ($F_{treatment} = 5.137$; $p < 0.05$; **Table 4.2**). In both ambient and elevated [CO_2] treatments, *I. spectablis* had the highest root mass of all tree species, followed closely by *A. pavonina*, though no difference in root mass was observed between *T. guayacan* and *T. rosea*. Similarly, root mass fraction in the elevated [CO_2] treatment was higher than in seedlings grown in the ambient treatment. Notably, *T. guayacan* seedlings in the elevated [CO_2] treatment had significantly higher root mass fractions compared to those in the ambient treatment. Furthermore, root:shoot ratios are higher in the elevated [CO_2] treatment compared to seedlings grown in the ambient treatment ($F_{Treatment} = 7.641$; $p < 0.05$). *T. guayacan* seedlings had an average root:shoot of 0.089 ± 0.021 in the elevated [CO_2] treatment

compared to 0.027 ± 0.007 in the ambient treatment. The two tree species associated with N_2-fixers: *I. spectablis* and *A. pavonina* notably had significantly higher total mass, root mass fractions, and root:shoot ratios than the two non-N_2 fixers: *T. guayacan* and *T.rosea* despite the lack of root nodulation.

Photosynthesis increased in some seedlings, but not in others

Photosynthesis averaged across all seedlings increased by ~ 20% in elevated [CO_2] ($F_{Treatment} = 5.100$; $p < 0.05$), though the strongest difference in photosynthetic activity was due to tree species identity (Ftree = 10.193; $p < 0.05$; **Table 4.3**). However, only *I. spectablis* and *T. rosea* had higher average photosynthesis in response to elevated [CO_2] (**Figure 4.2**), though not significantly in post-hoc tests ($Photosynthesis_{Elevated - Ambient} = 5.61$; $p = 0.79$ and $Photosynthesis_{Elevated - Ambient} = 16.50$; $p = 0.09$ respectively). In contrast, photosynthesis did not differ in *A. pavonina* seedlings between ambient and elevated [CO_2] treatments ($Photosynthesis_{Elevated - Ambient} = 0.50$; $p = 1.00$), and *T. guayacan* seedlings in the elevated [CO_2] treatment actually had lower photosynthesis than their counterparts in the ambient treatment, though the difference was insignificant ($Photosynthesis_{Elevated - Ambient} = -4.58$; $p = 0.99$).

Root phosphomonoesterase increased in A. pavonina and T. guayacan seedlings with elevated [CO_2]

Root phosphomonoesterase (PME) averaged across all seedlings increased in the elevated [CO_2] treatment ($F_{Treatment} = 7.081$; $p < 0.05$), though there was a strong influence of tree species identity ($F_{Tree:Treatment} = 6.050$; $p < 0.05$**; Table 4.4 A**). Root phosphomonoesterase (PME) increased in *A. pavonina* and *T. guayacan* but decreased in *T. rosea* and *I. spectablis* (**Table 4.4 B**; **Figure 4.3A**). In both treatments, *I. spectablis* and *A. pavonina* had the highest root PME activity compared to *T. guayacan* and *T. rosea*.

High root PME appeared to be negatively related to photosynthesis when average and root PME and photosynthesis in the elevated [CO_2] treatment was compared against average photosynthesis and root PME in the ambient treatment (**Figure 4.3B**). Values of E/A indicated that *A. pavonina* and *T. guayacan* seedlings, which significantly increased root PME in the elevated [CO_2] treatment also had reduced photosynthesis. *A. pavonina* had a root PME E/A value of 3.03 and *T. guayacan*, E/A = 3.14, which are higher than their E/A values for photosynthesis (0.47 and 0.89 respectively). In contrast, the opposite is true for *I. spectablis* and *T. rosea*.

Soil phosphomonoesterase and phosphodiesterase did not respond to elevated [CO_2]

Soil phosphomonoesterase (PME) and phosphodiesterase (PDE) did not differ with elevated [CO_2] ($F_{Treatment}$ = 2.608; p = 0.110; **Table 4.5 A, B**). All tree seedlings had markedly higher soil PME than soil PDE with the exception *of I. spectablis* (**Figure 4.4A, C**), which was lower than soil PME in *A. pavonina* (**Table 4.5 C**). There was no difference in soil PME between *T. guayacan* and *T. rosea*. Similarly, mean PDE activity was highest in *I. spectablis*, but this was not significantly different from *A. pavonina*. Likewise, both *T. rosea* and *T. guayacan* had similar soil PDE activity that was significantly lower when compared to *A. pavonina* and *I. spectablis*,

Soil PME and soil PDE were positively correlated (r = 0.48; p < 0.05; **Figure 4.4E**), with no apparent influence of the elevated [CO_2] treatment on the relationship. Similarly, there was no relationship between root PME and soil PME (r = 0.14; p = 0.18; **Figure 4.4B**). However, soil PDE was positively correlated with root PME (r = 0.59; p < 0.05; **Figure 4.4D**).

Root and soil phosphatase have different relationships to plant variables

Root PME was positively correlated with total biomass (r^2 = 0.38; p < 0.05; **Figure 4.5A**). Similarly, root PME was positively correlated with photosynthesis (r^2 = 0.27; p < 0.05; **Figure 4.5B**), though not with specific leaf area (SLA)(**Figure 4.5C)**. Although, soil PME had no correlation with total biomass (**Figure 4.5D**); soil PDE was positively correlated with total biomass (r^2 = 0.35; p < 0.05; **Figure 4.5G**). Soil PME had no correlation with photosynthesis (**Figure 4.5E**), but soil PDE was positively correlated with photosynthesis (r^2 = 0.29; p < 0.05; **Figure 4.5H**). Lastly, soil PME had no correlation with SLA (**Figure 4.5F**), and there was a significant, though slight negative correlation of soil PDE with SLA (**Figure 4.5I**).

Discussion

The CO_2 fertilization hypothesis assumes optimal light, water, and nutrients available to support increased plant growth in response to CO_2 (Thompson et al., 2019). However, concentrations of orthophosphate (P_i) in tropical forests are rarely optimal and can constrain growth despite increasing concentrations of CO_2 (Thompson et al., 2019). A key mechanism of enhancing P_i availability is the release of phosphatase enzymes that hydrolyze organic phosphorus compounds, releasing P_i as an end product. Here, we tested whether phosphatase enzymes in roots and soils would increase in response to elevated [CO_2] in tree seedlings of four

tropical tree species. We found that root phosphomonoesterase (PME) and root biomass, but not soil PME increased with elevated [CO_2]. Furthermore, the production of root PME in the elevated [CO_2] treatment appeared to correspond to lower photosynthetic activity.

Root mass fraction and root phosphomonoesterase increase with elevated [CO_2]

Increased below-ground allocation, particularly towards increasing root growth, is among the most consistent impacts of elevated [CO_2] (Norby et al., 1999; Iversen, 2010). Demand for nutrients is thought to be among the primary reasons for the consistent increase in belowground allocation in response to elevated [CO_2] (Wasaki et al., 2008), since roots and associated rhizosphere processes can influence soil nutrient pools (Niu et al., 2013). Seedlings in the elevated [CO_2] treatment had larger root systems on average than those in the ambient treatment (**Figure 4.1B**), which suggests that roots were proliferating in response for increased plant nutrient demand. Furthermore, we found that root PME activity was also higher in the elevated [CO_2] treatment. Root PME was on average higher in *A. pavonina* and *I. spectablis*, which are known to associate with N_2-fixers, compared to *T. guayacan* and *T. rosea*. consistent with a similar experiment (Nasto et al., 2019). Although root PME responded to elevated [CO_2], soil phosphatase did not.

Soil phosphatase was unresponsive to elevated [CO_2]

The positive response of root PME to elevated [CO_2] compared to the lack of response from soil phosphatase may be due to a closer association of root PME with plant demand. Acid phosphatase enzymes adhering to the root surface (root PME) and acid phosphatase enzymes from plants and microbial sources in soil (soil PME) are delineated according to where phosphatase activity is measured, rather than a known difference about their origin from a root or microbial cell (Skujins, 1978; Nannipieri et al., 2011). Although the origin of a particular phosphatase enzyme is difficult to trace, we consider root PME to be primarily of plant-origin and soil phosphatase to be primarily of microbial-origin, though it is likely that there is always a mix when either root or soil PME is measured. Regardless, soil phosphatase may not respond as strongly to elevated [CO_2] because changes in soil microbial function due to atmospheric and climatic change are likely indirect, occurring because of changes in plant inputs of litter or root exudates rather than a direct response to elevated [CO_2] (Kuzyakov et al., 2019). Since the seedlings were not producing litter, the lack of a strong response of soil phosphatase must be due

to the lack of changes in root exudation. Any changes in root exudation between seedlings in the ambient and elevated [CO_2] treatment was likely not strong enough to influence microbial-production of phosphatase enzymes. In contrast, phosphatase enzymes produced by roots, which largely adhere to the root surface (Tarafdar et al., 2001; Spohn et al., 2015), may reflect plant demand for P. For example, in an elevated [CO_2] study across a fertilization gradient, root phosphomonoesterase in *A. pavonina*, *I. spectablis*, and *T. guayacan* decreased in response to increasing N and P (Nasto et al., 2019). Furthermore, wheat grown aseptically in ambient and elevated [CO_2] conditions only increased root PME under P-deficiency, which was associated with a shoot P concentration < 0.18% (Barrett et al., 1998). Thus, the production and release of root PME under elevated [CO_2] is more likely than soil phosphatase to reflect increased plant demand for P.

Soil phosphodiesterase (PDE) and soil phosphomonoesterase (PME) act sequentially due to the nature of their substrates (Nannipieri et al., 2011; Reed et al., 2011). Soil PDE mineralizes organic P compounds containing two P_i-groups, such as phospholipids and nucleic acids, into phosphomonoesters. Phosphomonoesterase then releases Pi, making it available for plant or microbial uptake (Turner and Haygarth, 2005). As expected, soil PME and PDE are positively correlated (**Figure 4.4E**). However, there was no correlation between soil PME and any other measurements unlike soil PDE (**Figure 4.5**). Both root PME and soil PDE increased with total mass and photosynthesis across ambient and elevated [CO_2] treatments (**Figure 4.5A, B, G, H**). There was no effect of elevated [CO_2] on the correlations. Regardless, larger plants with higher rates of photosynthesis may require more P, thus requiring higher phosphatase activity. Soil PDE is positively correlated with root PME (**Figure 4.4D**), suggesting that microbes producing soil PDE first break down phosphodiesters before PME from roots completes mineralization to produce Pi. It is possible that the high production of PME from roots causes higher production of PDE from soil microbes since roots exude excessive amounts of PME (over a magnitude higher).

Root phosphomonoesterase and above-ground links

Globally photosynthesis is not yet saturated, indicating a strong potential for growth in forests if CO_2 concentrations increase (Millard et al., 2007). Certainly, photosynthesis reliably increases with rising [CO_2] (Ainsworth and Rogers, 2007), though whether this translates to increasing plant growth also depends on adequate root acquisition of water and nutrients

(Körner, 2006). Nitrogen and P can often limit plant growth (Fernández-Martínez et al., 2014b), though the lack of nodules on *A. pavonina* and *I. spectablis* seedlings suggest that N-limitation may not have been severe in our experiment. *A. pavonina* and *I. spectablis* seedlings formed nodules in a similar experiment (Nasto et al., 2019), though the growing medium contained a higher proportion of sand (95%) compared to our experiment (20%). In Nasto et al., (2019), foliar C:N, and C:P of *A. pavonina* and *I. spectablis* were lower than *T. guayacan* and the other non-N_2 fixer, *Swietenia macrophylla*, suggesting that A. pavonina and I. spectablis had more access to N and P in the elevated [CO_2] treatment. However, A. pavonina and T. guayacan both increased root PME in the elevated [CO_2] treatment, perhaps in response to plant demand for P.

We found that the increase in photosynthesis between ambient and elevated [CO_2] was really driven by only two species*: I. spectablis* and *T. rosea*, rather than uniformly in all seedlings in the elevated [CO_2] treatment. *A. pavonina* seedlings exhibited no change of photosynthesis between ambient and elevated [CO_2] treatments and *T. guayacan* seedlings decreased photosynthesis in the elevated [CO_2] treatment. However, *A. pavonina* and *T. guayacan* were the only tree species to increase root PME in response to elevated [CO_2], suggesting prioritization towards acquiring P.

Photosynthesis is regulated by the availability of light and substrates for ribulose-1,5-bisphosphate carboxylate/oxygenase enzyme (Rubisco), but also the capacity to use the carbon compounds produced (Paul and Pellny, 2003; Gamage et al., 2018). Photosynthesis creates sugars necessary for plant development of leaves, stems, and roots, but the rate at which these sugars are used to develop these "sinks" of carbon also requires an adequate supply of nutrients (Paul and Pellny, 2003). C and N metabolism is closely linked because Rubisco forms the bulk of N found in leaves and photosynthesis produces the carbon skeletons needed for amino acid synthesis. Thus, an insufficient supply of N could inhibit amino acid biosynthesis, causing an accumulation of glucose in leaves and down-regulating photosynthesis (Gamage et al., 2018). However, the links between C and P metabolism, particularly as related to photosynthesis are not as well defined. Datasets that analyze parameters of photosynthesis – Rubisco as represented by V_{cmax} and the electron transport chain as J_{max}) – within the context of foliar and soil N and P indicate that photosynthesis in plants are co-limited by N and P. However, there were stronger connections between P and J_{max} than N and J_{max}, suggesting that P may be influencing photosynthesis via P-rich metabolites (Norby et al., 2016). Photosynthesis could be limited by P

if the lack of inorganic P slows ribulose-1,5- bisphosphate (RuBP) regeneration. As a substrate for photosynthesis, low RuBP supply could inhibit photosynthesis, and this would appear as changes in J_{max} (Norby et al., 2016). Furthermore, inorganic P is used in many intermediate reactions and metabolites involved in creating sucrose for plant growth. If there is a lack of carbon sinks within the plant, and therefore decreased demand for sucrose synthesis, then Pi may not be as recycled quickly for use in photosynthesis. Ultimately, this could inhibit photosynthesis. Photosynthesis may not have increased in *A. pavonina* and *T. guayacan* because these seedlings had insufficient sink strength, perhaps due to P limitation. In a different experiment, *T. guayacan* was also found to have lower photosynthesis in elevated [CO_2] if nutrients were limiting (Trierweiler et al., 2018). In other words, P may not have limited photosynthesis directly, but rather slowed down the rate at which sucrose was used for new organs, causing a downregulation of photosynthesis.

Conclusion

The response of root functional traits to elevated [CO_2] may determine whether growth will be sustained, and therefore the size of the tropical C sink. Elevated [CO_2] tends to stimulate growth, assuming adequate supplies of water and nutrients. Thus, the capacity of the root system to adjust to meet plant demand will likely limit the extent of increased growth (BassiriRad et al., 2001). Previous studies have examined root form and function relative to P availability or tree identity, but it is important to consider that different tree species have variation in their physiological needs, which may be reflected in the way their roots traits respond to elevated [CO_2]. Our aim was to determine whether elevated [CO_2] increases root and microbial function how it relates to photosynthesis.

We found that root PME increased in elevated [CO_2] in certain tree species (*A. pavonina* and *T. guayacan*), but not others (*I. spectabilis* and *T. rosea*). Notably, photosynthesis in *A. pavonina* and *T. guayacan* seedlings did not increase with elevated [CO_2] as expected, suggesting a potential limitation to growth that may have inhibited photosynthetic activity. Although this study uses a limited amount of tree species, our results suggest that response to elevated [CO_2] may be tree species dependent, potentially due to tree species root or P utilization characteristics.

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Appendix

Table 4.1 Biomass, leaf area, and specific leaf area averaged across tree species and CO_2 . Error refers to standard error of the mean while E/A represents the average measurement of plants at elevated [CO_2] divided by the measurement of plants at ambient [CO_2].

	CO_2 Concentration			
	Ambient	Elevated	E/A	p-value
Adenanthera pavonina				
Total biomass (g)	0.730 ± 0.055	0.725 ± 0.053	0.993	n.s.
Leaves (g)	0.438 ± 0.029	0.422 ± 0.032	0.963	n.s.
Stems (g)	0.201 ± 0.014	0.197 ± 0.015	0.980	n.s.
Roots (g)	0.091 ± 0.015	0.107 ± 0.014	1.176	n.s.
Root:shoot (g g^{-1})	0.136 ± 0.019	0.171 ± 0.016	1.257	n.s.
Leaf area (m^2)	104.46 ± 6.189	75.221 ± 5.524	0.720	n.s.
Specific leaf area (m^2 g^{-1})	240.472 ± 6.575	179.077 ± 3.624	0.745	< **0.05**
Root mass fraction (%)	11.74 ± 1.56	14.45 ± 1.19	1.23	n.s.
Inga spectablis				
Total biomass (g)	2.369 ± 0.148	2.811 ± 0.119	1.187	n.s.
Leaves (g)	1.356 ± 0.084	1.634 ± 0.073	1.205	n.s.
Stems (g)	0.621 ± 0.045	0.677 ±0.029	1.090	n.s.
Roots (g)	0.337 ± 0.043	0.501 ± 0.053	1.487	n.s.
Root:shoot (g g^{-1})	0.180 ± 0.017	0.217 ± 0.023	1.206	n.s.
Leaf area (m^2)	211.742 ± 14.240	221.619 ± 10.400	1.047	n.s.
Specific leaf area (m^2 g^{-1})	156.235 ± 3.781	135.838 ± 2.675	0.869	n.s.
Root mass fraction (%)	15.07 ± 1.27	17.56 ± 1.45	1.17	n.s.

Table 4.1 continued

	CO_2 Concentration			
	Ambient	Elevated	E/A	p-value
Tabebuia guayacan				
Total biomass (g)	0.249 ± 0.035	0.158 ± 0.039	0.719	n.s
Leaves (g)	0.167 ± 0.026	0.102 ± 0.019	0.611	n.s.
Stems (g)	0.076 ± 0.009	0.062 ± 0.007	0.816	n.s.
Roots (g)	0.006 ± 0.002	0.015 ± 0.005	2.500	< **0.05**
Root:shoot (g g^{-1})	0.027 ± 0.007	0.089 ± 0.021	3.296	< **0.05**
Leaf area (m^2)	41.258 ± 6.834	21.118 ± 3.479	0.512	n.s.
Specific leaf area (m^2 g^{-1})	244.777 ± 5.926	212.047 ± 12.511	0.866	n.s.
Root mass fraction (%)	2.54 ± 0.63	7.85 ± 1.58	3.09	< **0.05**
Tabebuia rosea				
Total biomass (g)	0.272 ± 0.036	0.267 ± 0.035	0.982	n.s.
Leaves (g)	0.137 ± 0.022	0.132 ± 0.023	0.964	n.s.
Stems (g)	0.115 ± 0.013	0.121 ± 0.013	1.052	n.s.
Roots (g)	0.010 ± 0.002	0.014 ± 0.003	1.400	n.s.
Root:shoot (g g^{-1})	0.041 ± 0.009	0.053 ± 0.010	1.293	n.s.
Leaf area (m^2)	27.530 ± 5.272	21.552 ± 4.259	0.783	n.s.
Specific leaf area (m^2 g^{-1})	195.015 ± 6.200	159.218 ± 9.319	0.816	< **0.05**
Root mass fraction (%)	3.91 ± 0.79	4.94 ± 0.84	1.26	n.s.

Table 4.2 ANOVA tables for total biomass (A), leaf (B), stem (C), root (D), and root mass fraction (E).

A. Total biomass ANOVA

	Sum of Squares	Degrees of Freedom	F-value	p-value
Tree Species	33110	3	31.053	< **0.05***
Treatment	1237	1	3.481	0.065
Tree Species: Treatment	4295	3	4.028	< **0.05***
Residuals	305	86		

Table 4.2 continued

B. Leaf biomass ANOVA

	Sum of Squares	Degrees of Freedom	F-value	p-value
Tree Species	56724	3	140.494	< **0.05***
Treatment	166	1	1.235	0.270
Tree Species: Treatment	744	3	1.842	0.146
Residuals	11574	86		

C. Stem biomass ANOVA

	Sum of Squares	Degrees of Freedom	F-value	p-value
Tree Species	558006	3	152.780	< **0.05***
Treatment	10	1	0.081	0.777
Tree Species: Treatment	301	3	0.792	0.502
Residuals	10884	86		

D. Root biomass ANOVA

	Sum of Squares	Degrees of Freedom	F-value	p-value
Tree Species	29047	3	30.382	< **0.05***
Treatment	5050	1	15.847	< **0.05***
Tree Species: Treatment	7673	3	8.026	< **0.05***
Residuals	27407	86		

Table 4.2 continued

E. Root mass fraction ANOVA

	Sum of Squares	Degrees of Freedom	F-value	p-value
Tree Species	29047	3	30.382	< **0.05***
Treatment	5050	1	15.847	< **0.05***
Tree Species: Treatment	7673	3	8.026	< **0.05***
Residuals	27407	86		

Table 4.3 Photosynthesis ANOVA table.

	Sum of Squares	Degrees of Freedom	F-value	p-value
Tree Species	2115.2	3	10.193	< **0.05***
Treatment	41.5	1	0.600	0.444
Tree Species: Treatment	1300.7	3	6.268	< **0.05***
Residuals	2282.6	33		

Table 4.4 Root phosphomonoesterase (PME) ANOVA table (A) and summary (B).

A. ANOVA table

	Sum of Squares	Degrees of Freedom	F-value	p-value
Tree Species	59177	4	113.161	< **0.05***
Treatment	900	1	6.887	< **0.05***
Tree Species: Treatment	2399	4	4.587	< **0.05***
Residuals	11243	86		

Table 4.4 continued

B. Means, SEM, and SD table

Tree Species	Treatment	Root PME average (μmol pNP g_{rootDW}^{-1})	Root PME SEM	Root PME SD
ADEPAV	Ambient	55.3336	5.1234	16.9926
ADEPAV	ElevatedCO_2	120.9355	19.4534	64.5197
INGA	Ambient	212.7083	26.4768	91.7184
INGA	ElevatedCO_2	182.0408	11.2067	38.8211
TABGUA	Ambient	42.3333	7.3562	25.4826
TABGUA	ElevatedCO_2	79.1833	15.7809	54.6665
TABROS	Ambient	10.8900	1.2970	4.4930
TABROS	ElevatedCO_2	9.1783	1.6635	5.7625

Table 4.5 Soil phosphomonoesterase (PME) ANOVA table (A), phosphodiesterase (B), and summary (C).

A. Soil PME ANOVA table

	Sum of Squares	Degrees of Freedom	F-value	p-value
Tree Species	21812	4	9.759	< **0.05***
Treatment	1457	1	2.608	0.110
Tree Species: Treatment	2393	4	1.071	0.376
Residuals	48055	86		

Table 4.5 continued

B. Soil PDE ANOVA table

	Sum of Squares	Degrees of Freedom	F-value	p-value
Tree Species	32598	4	17.467	< **0.05***
Treatment	176	1	0.377	0.541
Tree Species: Treatment	820	4	0.439	0.780
Residuals	40125	86		

C. Summary of soil PME and PDE across tree species and treatments

Tree Species	Treatment	Soil PME average (nmol MU g_{soilDW}^{-1})	Soil PME SEM	Soil PME SD
ADEPAV	Ambient	206.2000	23.4138	77.6548
ADEPAV	ElevatedCO_2	244.3327	36.6270	121.4781
INGA	Ambient	111.9692	9.5479	33.0750
INGA	ElevatedCO_2	180.5617	74.3277	257.4786
TABGUA	Ambient	109.4967	6.4632	22.3893
TABGUA	ElevatedCO_2	147.2892	21.7944	75.4982
TABROS	Ambient	130.5436	10.4157	34.5450
TABROS	ElevatedCO_2	145.0692	11.1847	38.7450

Table 4.5 continued

Tree Species	Treatment	Soil PDE average (nmol MU gsoilDW-1)	Soil PDE SEM	Soil PDE SD
ADEPAV	Ambient	37.5209	23.4138	14.4154
ADEPAV	ElevatedCO_2	44.7718	36.6270	18.9670
INGA	Ambient	72.1233	9.5479	34.0623
INGA	ElevatedCO_2	78.3425	74.3277	51.8599
TABGUA	Ambient	30.3242	6.4632	10.2044
TABGUA	ElevatedCO_2	26.9100	21.7944	11.9825
TABROS	Ambient	22.0118	10.4157	5.4209
TABROS	ElevatedCO_2	26.3783	11.1847	6.8165

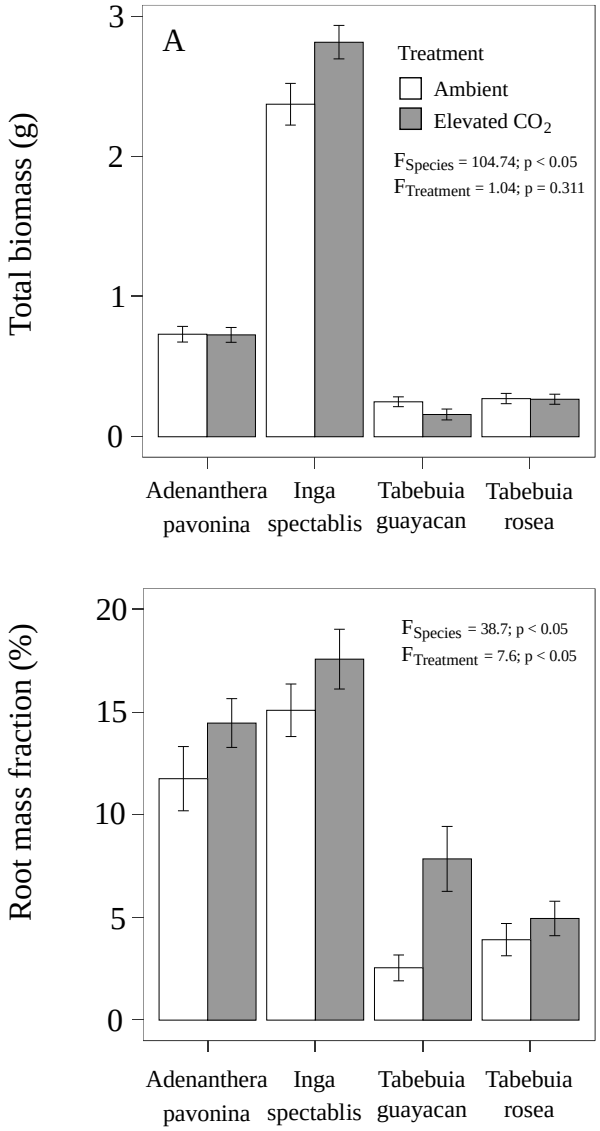

Figure 4.1 Average plant biomass in each tree species and treatment (A). Root mass biomass in each tree species and treatment (B). Error bars represent standard error of the mean.

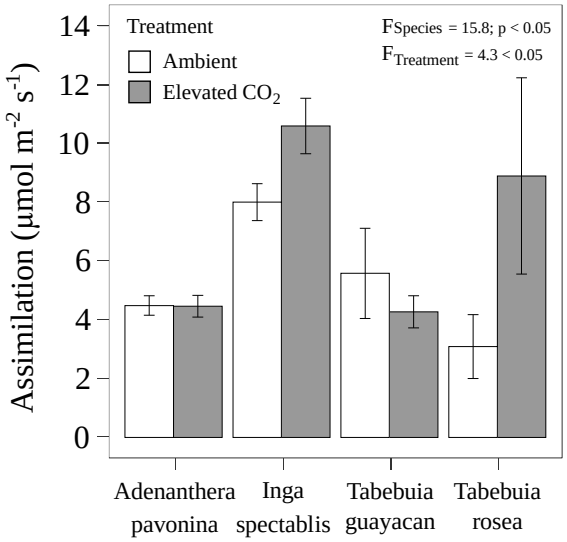

Figure 4.2 Photosynthesis by tree species and CO2 treatment. Error bars represent standard error of the mean.

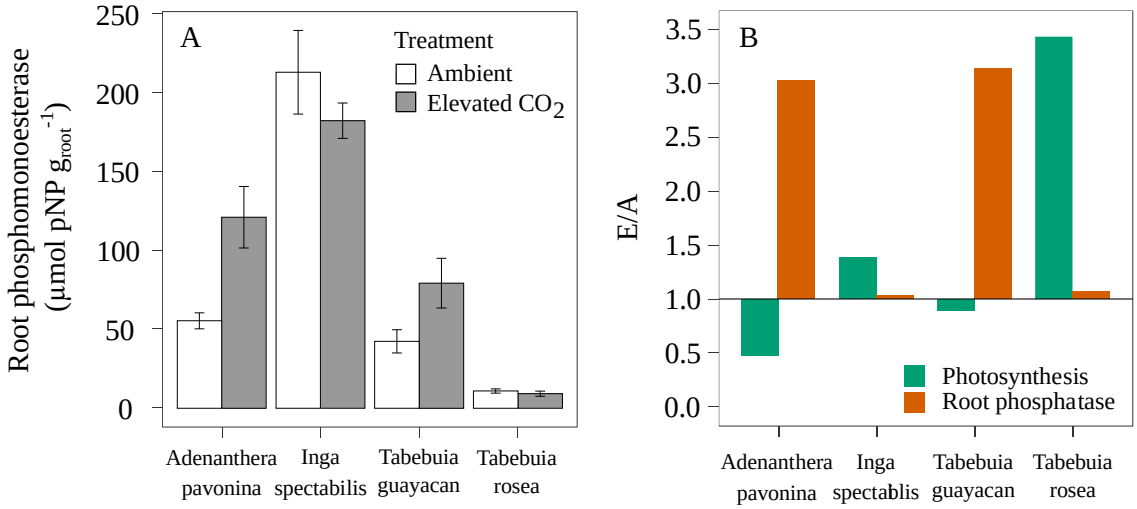

Figure 4. 3 Root phosphomonoesterase by tree species and CO_2 (A). Error bars represent standard error of the mean. Elevated/Ambient plot representing average activity at the elevated [CO_2] treatment compared to the ambient treatment of photosynthesis and root phosphomonoesterase (B)

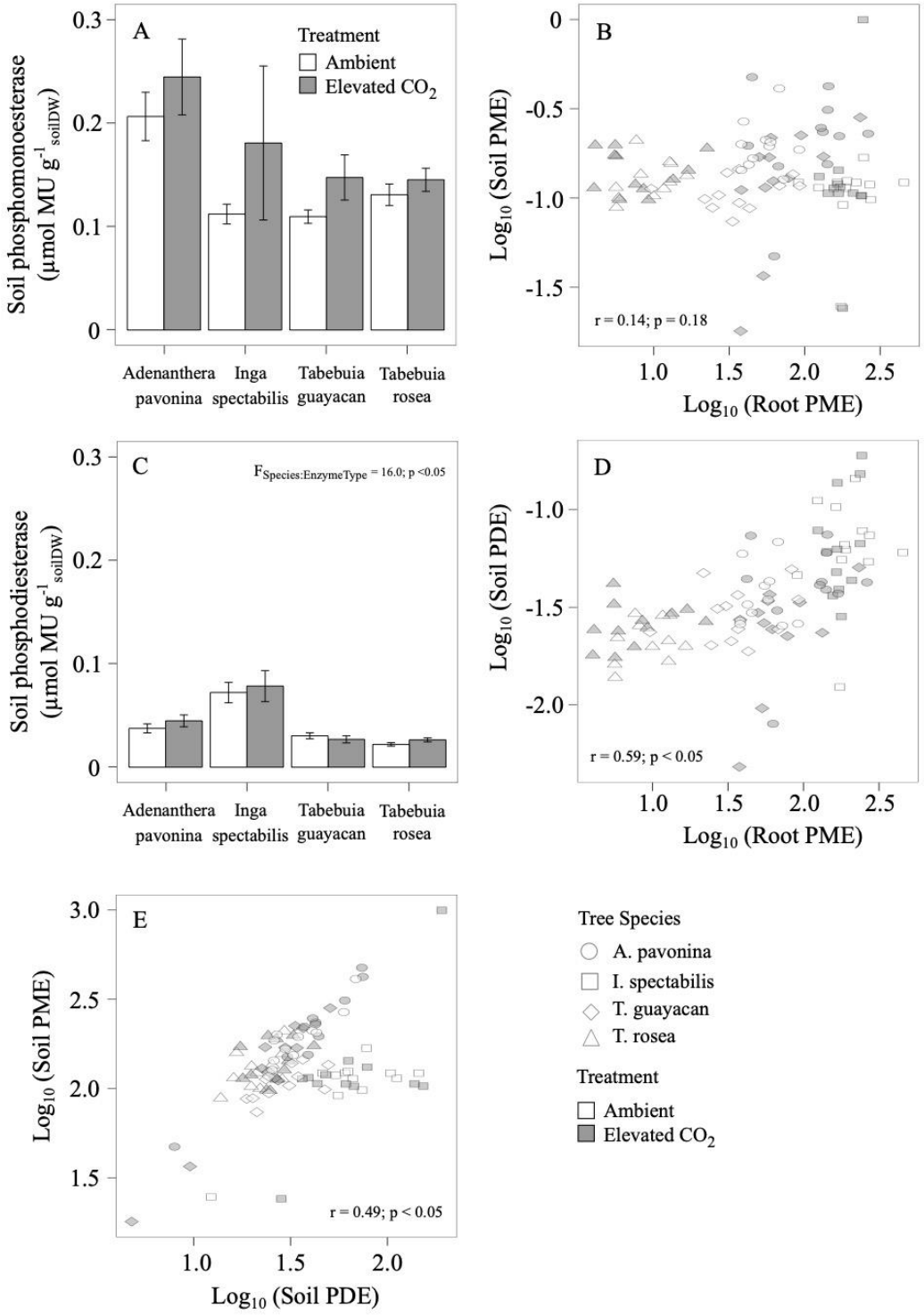

Figure 4. 4 Average soil phosphomonoesterase (PME) (A) and phosphodiesterase (PDE) (C) across tree species and CO_2 treatments. Error bars represent standard error of the mean. Correlations between root phosphomonoesterase (PME) and soil PME (B), soil PDE (D). Correlation between soil PDE and PME (E).

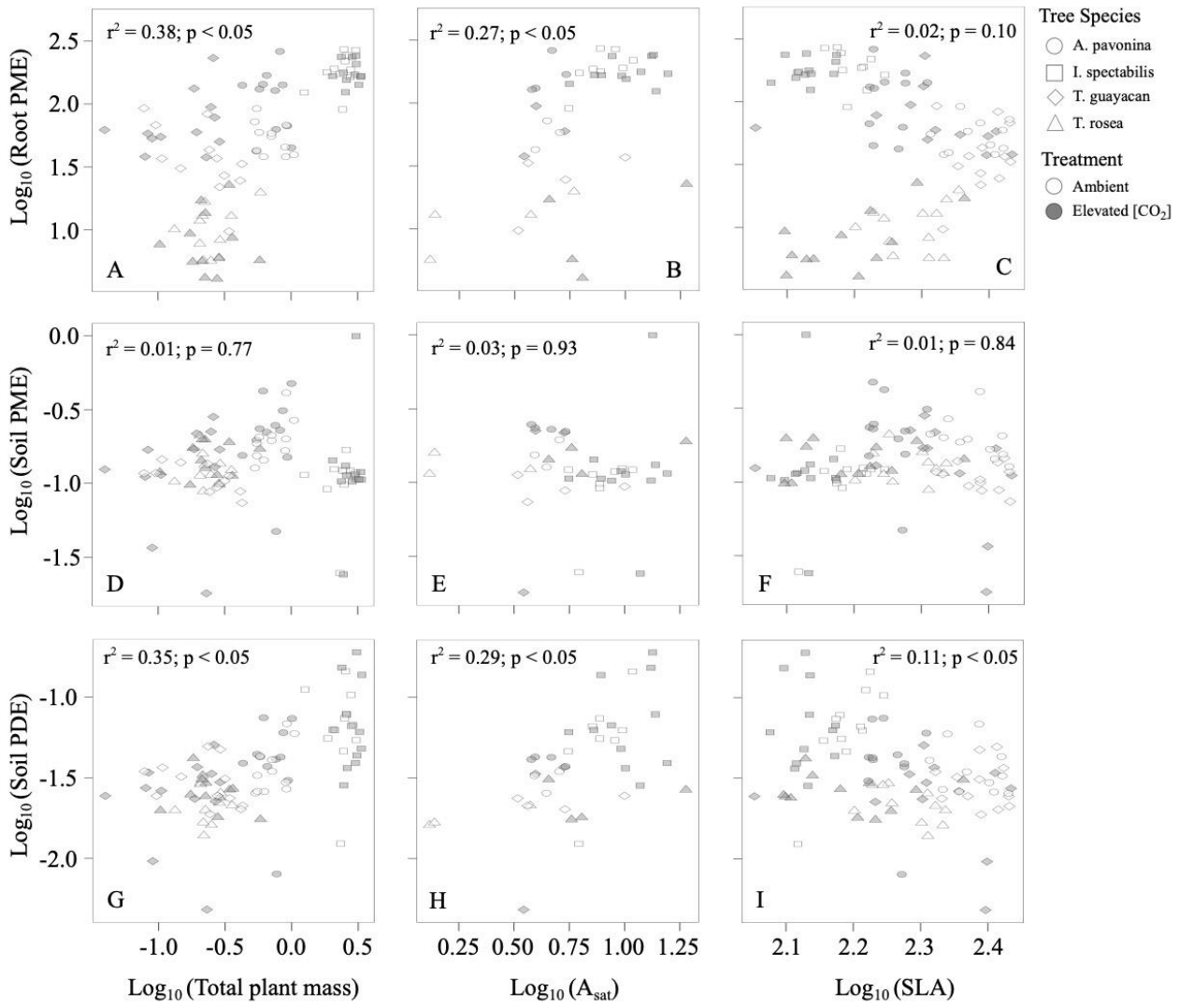

Figure 4. 5 Correlations of root PME, soil PME, and soil PDE with total plant mass (left column), A_{sat} (middle column), and specific leaf area (SLA; right column).

Chapter 5

Conclusion

The response of tropical forests to atmospheric and climatic change is inherently influenced by the roots and the rhizosphere, which regulate, in part, P availability and acquisition. Phosphatase in roots and soil is a critical facet of organic P mineralization, particularly in the tropics where available P occurs in much more limited concentrations relative to temperate ecosystems. Understanding the factors that influence phosphatase activity, such as how it varies according to differing P availabilities, tree species, microbial communities, and soil depth is an important aspect of improving our understanding of the phosphorus cycle, and the capacity of phosphatase to regulate availability of P for plant growth. Ultimately, quantifying, characterizing, and accurately modeling phosphatase in tropical soils is imperative to capturing the nature of tropical forest function and composition because variation in phosphatase activity may reflect different degrees of access to the soil organic P pool and therefore the extent of P availability and limitation for different tree species.

Phosphatase activity in the rhizosphere of different tree species

Earlier work has suggested that phosphatase is an adaptive enzyme that is heavily influenced by plant phosphorus demand (Tarafdar and Jungk, 1987), and that different tree species may exhibit different patterns of phosphatase that correspond to the different types of organic phosphorus-compounds targeted (Turner, 2008). However, rarely considered in this plant-soil perspective is the role of microbial symbionts within the rhizosphere.

Phosphatase in the rhizosphere is a mix of both root- and microbial-derived enzymes, with no methods currently available to distinguish one from the other. Whereas alkaline phosphatase is considered exclusive to microbes, acid phosphatase is expressed by both (P Nannipieri et al., 2011). The microbial community surrounding the plant root is an equally important facet of understanding how plants access available and organic P with numerous studies indicating the role of microbially-produced hormones in altering root morphology (Taylor-Teeples et al., 2016; Wei and Li, 2016), microbial aid with solubilization (Gururani et al., 2013; Ramachandran et al., 2007; Saghir et al., 2014), and of course, mycorrhizal symbiosis that directly contributes to scavenging the soil volume for soil phosphorus (Bergmann et al., 2017; Shi et al., 2017; Walder and Van Der Heijden, 2015). Our study shows that increased phosphatase activity in bacterial colonies isolated from the rhizosphere of tree species corresponds to tree species phosphatase activity (Cabugao et al. 2017). Variation in the function of the rhizosphere as central hub of interactions between soil, microbe, and plant may help

explain different P acquisition strategies and therefore trees species distribution and function in the phosphorus cycle.

Phosphatase activity and root traits

As sessile organisms, plant survival depends on the plasticity of morphological, physiological, and functional characteristics - functional traits- to cope with changes in abiotic and biotic conditions (Violle et al., 2007). Thus, understanding plant functional traits is nothing short of understanding why plants exist where they do and how they interact with the environment in which they grow (Reich, 2014). The development of a leaf economic spectrum (LES) was a crucial tool in understanding the tradeoff between plant growth and survival across different ecosystems, specifically because LES traits could link photosynthesis to water use and nutrient uptake (Wright et al., 2004). However, a corresponding root economic spectrum (RES) to explain the influence of belowground traits on patterns of plant growth across the globe remains elusive (Kramer-Walter et al., 2016; Mommer and Weemstra, 2012), likely because roots serve many functions (anchorage, storage, symbiotic interactions, and resource uptake) that make it difficult to place traits in single spectrum (Kramer-Walter et al., 2016). However, understanding the connection between root morphology and function is critical to understanding belowground function.

Phosphorus cycling in tropical forests is characterized as a very tight system, where the main input is leaf litter and the main process of replenishing available P is the mineralization of organic P by phosphatase enzymes (Darch et al., 2016; Reed et al., 2011; Benjamin L. Turner and Engelbrecht, 2011). Representing each tree species and their influence on phosphorus mineralization would be an insurmountable task, but there may be certain root traits and soil factors that regulate phosphatase that can enable the derivation of meaningful patterns and predictions across a wide range of species and forests. We found that SRL and fine-root mass density were critically important to both soil and root PME and derived multiple linear regression equation that we hope we can apply to more sites to potentially improve how biochemical mineralization is represented.

Phosphatase activity and elevated [CO_2]

A major uncertainty in understanding and modeling how tropical forests may change in future climates is the capacity to which roots can acquire P necessary to increasing growth. Increased belowground carbon allocation is a consistent response in elevated [CO_2] studies,

presumably to enhance acquisition of water and nutrients (Wasaki et al., 2005). Indeed, under elevated [CO_2], root branching, fine-root production, and root extension increase in order to enhance the absorptive area of roots for resources such as P (Barrett et al., 1998). Furthermore, in tropical soils that are P-limited, root P acquisition has the potential to influence soil P pools, primarily through altering the soil organic and available P pools (Jin et al., 2013). This was shown in the rhizosphere of cereal crops, where organic P fractions in the rhizosphere were altered in elevated [CO_2] (Jin et al., 2013).

Our study suggests that phosphatase may be upregulated in some tree species, but not others. Phosphatase activity under elevated [CO_2] is likely a key facet of meeting increased P demand by the mineralization of the organic P pool (Barrett 1998). Our findings do suggest that phosphatase activity can increase in response to elevated [CO_2] and further foliar analysis may yield further insights into whether we can attribute differences in phosphatase activity to differing levels of phosphorus demand. If phosphatase is highly upregulated in some tree species as opposed to others, it could mean an advantage in elevated [CO_2] and potentially a shift in forest composition.

Root exploration and intensity of investment in the soil volume

Root traits shape plant P acquisition by regulating root interactions in the soil volume, such as how extensive the root system is and how intensely soils within the vicinity of the root surface are mined for available P. Efforts to distill root traits into meaningful patterns that correspond to conservative or acquisitive life strategies have primarily focused on root traits analogous to leaf traits in the leaf economic spectrum (Weemstra et al., 2016), root diameter (Kong et al., 2017, 2014), and arbuscular mycorrhizal colonization trade-offs (Comas et al., 2014). However, where might phosphatase activity fit in light of these traits? I propose that while some root traits are effective to increasing soil exploration for available P, there are other root traits, such as phosphatase activity, that increase the intensity to which rhizosphere soil is mined for P (**Figure 5.1** in the Appendix).

Phosphatase produced from roots and microbes in the rhizosphere tend to remain close to the root surface (Yadav and Tarafdar, 2001). Root phosphatase activity varied with tree species identity (Cabugao et al., 2017), through differences in root morphology or root distribution (**Figure 3.7**). While arbuscular mycorrhizal fungi (AMF) are important to soil exploration for inorganic phosphorus (P_i) (Clark and Zeto, 2000), phosphatase activity could be viewed as a

mechanism to intensely acquire P from a given soil volume. Thus, phosphatase activity represents a focus on localized sources of P as opposed to symbiotic interactions with AMF, which favors exploration of distant sources of available P. Essentially, roots have multiple ways of being acquisitive depending on how intensely local sources of P are utilized as opposed to distant sources (**Figure 5.1**).

The quadrants in **Figure 5.1** correspond to variations in root traits depending on the reliance on soil exploration or thoroughly using local sources of P. We found a strong positive correlation between specific root length, a root trait commonly associated with soil exploration (Richardson et al., 2009), and root phosphatase, considered important to accessing soil organic P within the rhizosphere (Lambers et al., 2006). These two traits would occur in quadrant I of **Figure 5.1**. Quadrant II would contain plants that either form low or no symbiotic interactions with AMF but do intensely use local sources of P, such as cluster roots that rely on carboxylate exudation (Lambers et al., 2008). Quadrant III represents root systems that minimally explore the soil volume or do not heavily invest in phosphatase activity. These may be plants that grow slowly or have high phosphorus use efficiency relative to other plants in the other quadrants. Alternatively, plants in Quadrant III may occur in close proximity to plants with more acquisitive traits, opportunistically acquiring P released by other plants. Lastly, quadrant IV would describe plants that have high rates of mycorrhizal colonization, but not necessarily high amounts of phosphatase or carboxylate exudation. For example, there is a general trend that high phosphatase activity corresponds to lower AMF colonization (Nasto et al., 2019), though a positive correlation between AMF colonization and phosphatase has been observed (Dodd et al., 1987). While further work remains to confirm this framework, it does place root phosphatase within the context of more commonly measured root traits, building on efforts to describe patterns of P acquisition by including a root trait that maximizes not the size of the root system, but the influence of roots on organic P in rhizosphere soil.

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Appendix

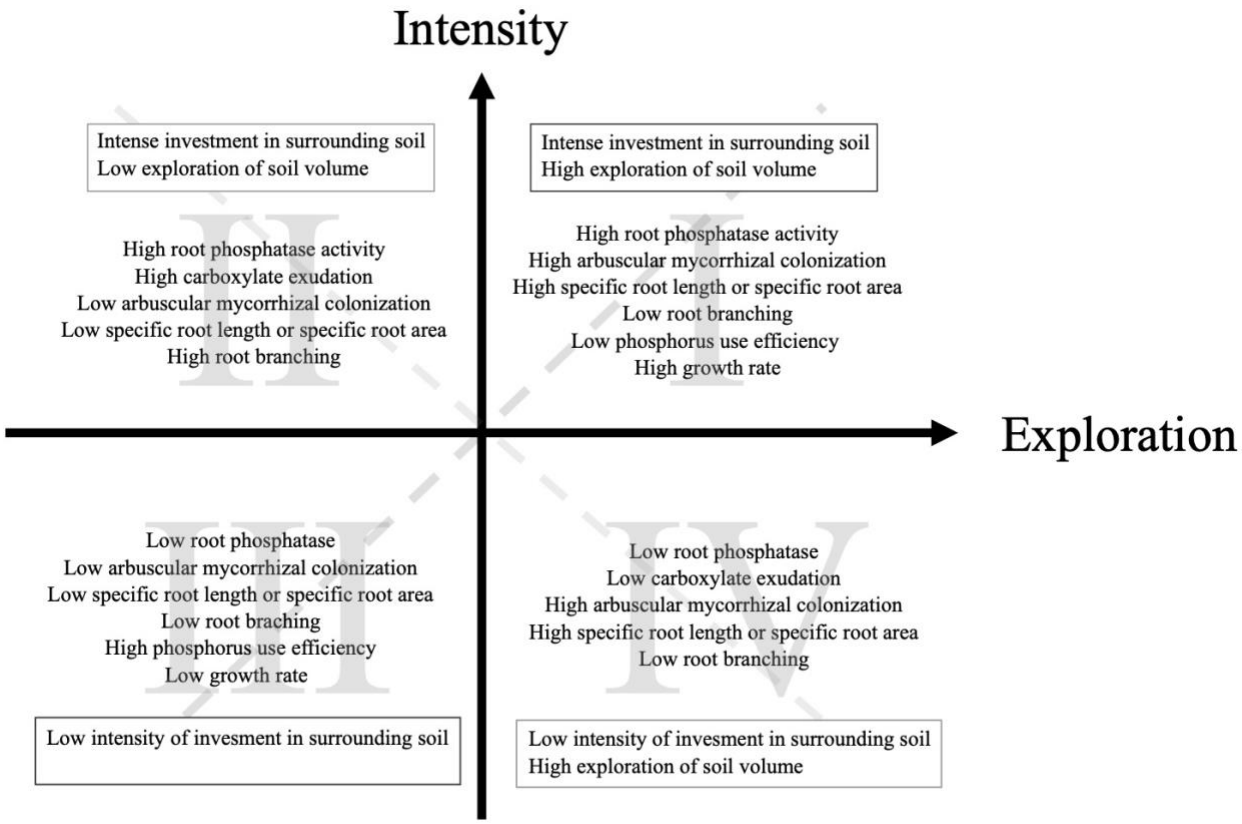

Figure 5. 1 Root exploration and intensity of investment in a given soil volume.

Vita

Kristine Grace Manno Cabugao was born in Kapangan, Luzon, Philippines and immigrated to Sunnyvale, California with her family when she was six years old. In 2014 she received her Bachelor of Science degree from University of California Berkeley and subsequently moved to Knoxville, Tennessee to pursue her graduate studies. As an ecologist with a background in molecular biology she is most interested in unraveling how nutrient cycling in the rhizosphere corresponds to plant growth and how variation in nutrient acquisition regulates, in part, forest composition and function. With a strong interest in the intersection between society, policy, and the environment, she hopes to pursue research that directly contributes to sustainable development, conservation, or ecological restoration.